(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: BIODEGRADABLE POLY(BETA-AMINO ESTERS) AND USES THEREOF
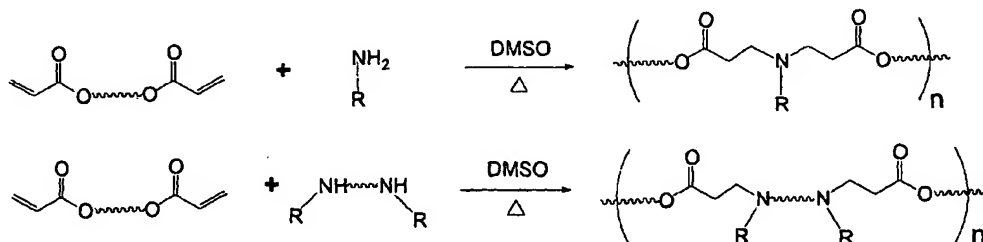
(57) Abstract: Poly(β-amino esters) prepared from the conjugate addition of bis(secondary amines) or primary amines to a bis(acrylate ester) are described. Methods of preparing these polymers from commercially available starting materials are also provided. These tertiary amine-containing polymers are preferably biodegradable and biocompatible and may be used in a variety of drug delivery systems. Given the poly(amine) nature of these polymers, they are particularly suited for the delivery of polynucleotides. Nanoparticles containing polymer/polynucleotide complexes have been prepared. The inventive polymers may also be used to encapsulate other agents to be delivered. They are particularly useful in delivering labile agents given their ability to buffer the pH of their surroundings. A system for preparing and screening polymers in parallel using semi-automated robotic fluid delivery systems is also provided.

BIODEGRADABLE POLY(BETA-AMINO ESTERS) AND USES THEREOF

Government Support

Background of the Invention

The treatment of human diseases through the application of nucleotide-based drugs such as DNA and RNA has the potential to revolutionize the medical field

15   (Anderson *Nature* 392(Suppl.):25-30, 1996; Friedman *Nature Med.* 2:144-147, 1996; Crystal *Science* 270:404-410, 1995; Mulligan *Science* 260:926-932, 1993; each of which is incorporated herein by reference). Thus far, the use of modified viruses as gene transfer vectors has generally represented the most clinically successful approach to gene therapy. While viral vectors are currently the most efficient gene transfer

20   agents, concerns surrounding the overall safety of viral vectors, which include the potential for unsolicited immune responses, have resulted in parallel efforts to develop non-viral alternatives (for leading references, see: Luo *et al. Nat. Biotechnol.* 18:33-37,2000; Behr *Acc. Chem. Res.* 26:274-278, 1993; each of which is incorporated herein by reference). Current alternatives to viral vectors include polymeric delivery systems

25   (Zauner *et al. Adv. Drug Del. Rev.* 30:97-113, 1998; Kabanov *et al. Bioconjugate Chem.* 6:7-20, 1995; each of which is incorporated herein by reference), liposomal formulations (Miller *Angew. Chem. Int. Ed.* 37:1768-1785, 1998; Hope *et al. Molecular Membrane Technology* 15:1-14, 1998; Deshmukh *et al. New J. Chem.* 21:113-124, 1997; each of which is incorporated herein by reference), and "naked" DNA injection

30   protocols (Sanford *Trends Biotechnol.* 6:288-302, 1988; incorporated herein by reference). While these strategies have yet to achieve the clinical effectiveness of viral vectors, the potential safety, processing, and economic benefits offered by these

methods (Anderson *Nature* 392(Suppl.):25-30, 1996; incorporated herein by reference) have ignited interest in the continued development of non-viral approaches to gene therapy (Boussif *et al. Proc. Natl. Acad. Sci. USA* 92:7297-7301, 1995; Putnam *et al. Macromolecules* 32:3658-3662, 1999; Lim *et al. J. Am. Chem. Soc.* 121:5633-5639,

5   1999; Gonzalez *et al. Bioconjugate Chem.* 10:1068-1074, 1999; Kukowska-Latallo *et al. Proc. Natl. Acad. Sci. USA* 93:4897-4902, 1996; Tang *et al. Bioconjugate Chem.* 7:703-714, 1996; Haensler *et al. Bioconjugate Chem.* 4:372-379, 1993; each of which is incorporated herein by reference).

Cationic polymers have been widely used as transfection vectors due to the

10   facility with which they condense and protect negatively charged strands of DNA. Amine-containing polymers such as poly(lysine) (Zauner *et al. Adv. Drug Del. Rev.* 30:97-113, 1998; Kabanov *et al. Bioconjugate Chem.* 6:7-20, 1995; each of which is incorporated herein by reference), poly(ethylene imine) (PEI) (Boussif *et al. Proc. Natl. Acad. Sci. USA* 92:7297-7301, 1995; incorporated herein by reference), and

15   poly(amidoamine) dendrimers (Kukowska-Latallo *et al. Proc. Natl. Acad. Sci. USA* 93:4897-4902, 1996; Tang *et al. Bioconjugate Chem.* 7:703-714, 1996; Haensler *et al. Bioconjugate Chem.* 4:372-379, 1993; each of which is incorporated herein by reference) are positively-charged at physiological pH, form ion pairs with nucleic acids, and mediate transfection in a variety of cell lines. Despite their common use, however,

20   cationic polymers such as poly(lysine) and PEI can be significantly cytotoxic (Zauner *et al. Adv. Drug Del. Rev.* 30:97-113, 1998; Deshmukh *et al. New J. Chem.* 21:113-124, 1997; Choksakulnimitr *et al. Controlled Release* 34:233-241, 1995; Brazeau *et al. Pharm. Res.* 15:680-684, 1998; each of which is incorporated herein by reference). As a result, the choice of cationic polymer for a gene transfer application generally

25   requires a trade-off between transfection efficiency and short- and long-term cytotoxicity. Additionally, the long-term biocompatibility of these polymers remains an important issue for use in therapeutic applications *in vivo*, since several of these polymers are not readily biodegradable (Uhrich *Trends Polym. Sci.* 5:388-393, 1997; Roberts *et al. J. Biomed. Mater. Res.* 30:53-65, 1996; each of which is incorporated

30   herein by reference).

In order to develop safe alternatives to existing polymeric vectors and other functionalized biomaterials, degradable polyesters bearing cationic side chains have

been developed (Putnam *et al. Macromolecules* 32:3658-3662, 1999; Barrera *et al. J. Am. Chem. Soc.* 115:11010-11011, 1993; Kwon *et al. Macromolecules* 22:3250-3255, 1989; Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; Zhou *et al. Macromolecules* 23:3399-3406, 1990; each of which is incorporated herein by reference). Examples of

5   these polyesters include poly(L-lactide-co-L-lysine) (Barrera *et al. J. Am. Chem. Soc.* 115:11010-11011, 1993; incorporated herein by reference), poly(serine ester) (Zhou *et al. Macromolecules* 23:3399-3406, 1990; each of which is incorporated herein by reference), poly(4-hydroxy-L-proline ester) (Putnam *et al. Macromolecules* 32:3658-3662, 1999.; Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; each of which is

10   incorporated herein by reference), and more recently, poly[α-(4-aminobutyl)-L-glycolic acid]. Poly(4-hydroxy-L-proline ester) and poly[α-(4-aminobutyl)-L-glycolic acid] were recently demonstrated to condense plasmid DNA through electrostatic interactions, and to mediate gene transfer (Putnam *et al. Macromolecules* 32:3658-3662, 1999; Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; each of which is

15   incorporated herein by reference). Importantly, these new polymers are significantly less toxic than poly(lysine) and PEI, and they degrade into non-toxic metabolites. It is clear from these investigations that the rational design of amine-containing polyesters can be a productive route to the development of safe, effective transfection vectors. Unfortunately, however, present syntheses of these polymers require either the

20   independent preparation of specialized monomers (Barrera *et al. J. Am. Chem. Soc.* 115:11010-11011, 1993; incorporated herein by reference), or the use of stoichiometric amounts of expensive coupling reagents (Putnam *et al. Macromolecules* 32:3658-3662, 1999; incorporated herein by reference). Additionally, the amine functionalities in the monomers must be protected prior to polymerization (Putnam *et al. Macromolecules*

25   32:3658-3662, 1999; Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; Gonzalez *et al. Bioconjugate Chem.* 10:1068-1074, 1999; Barrera *et al. J. Am. Chem. Soc.* 115:11010-11011, 1993; Kwon *et al. Macromolecules* 22:3250-3255, 1989; each of which is incorporated herein by reference), necessitating additional post-polymerization deprotection steps before the polymers can be used as transfection agents.

30   There exists a continuing need for non-toxic, biodegradable, biocompatible polymers that can be used to transfect nucleic acids and that are easily prepared efficiently and economically. Such polymers would have several uses, including the

delivery of nucleic acids in gene therapy as well as in the packaging and/or delivery of diagnostic, therapeutic, and prophylactic agents.

Summary of the Invention

5   The present invention provides polymers useful in preparing drug delivery devices and pharmaceutical compositions thereof. The invention also provides methods of preparing the polymers and methods of preparing microspheres and other pharmaceutical compositions containing the inventive polymers.

The polymers of the present invention are poly(β-amino esters) and salts and
10   derivatives thereof. Preferred polymers are biodegradable and biocompatible. Typically, the polymers have one or more tertiary amines in the backbone of the polymer. Preferred polymers have one or two tertiary amines per repeating backbone unit. The polymers may also be co-polymers in which one of the components is a poly(β-amino ester). The polymers of the invention may readily be prepared by
15   condensing bis(secondary amines) or primary amines with bis(acrylate esters). A polymer of the invention is represented by either of the formulae below:
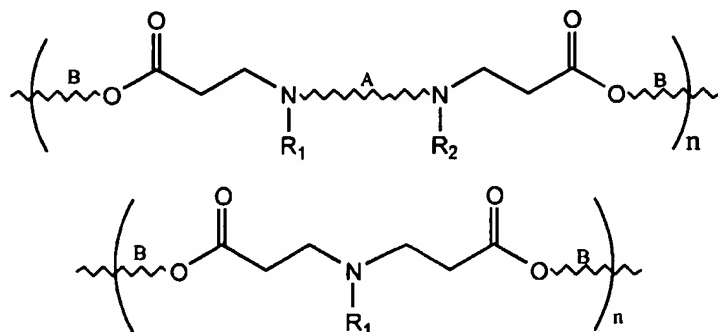
where A and B are linkers which may be any substituted or unsubstituted, branched or
20   unbranched chain of carbon atoms or heteroatoms. The molecular weights of the polymers may range from 1000 g/mol to 20,000 g/mol, preferably from 5000 g/mol to 15,000 g/mol. In certain embodiments, B is an alkyl chain of one to twelve carbons atoms. In other embodiments, B is a heteroaliphatic chain containing a total of one to twelve carbon atoms and heteroatoms. The groups R_1 and R_2 may be any of a wide
25   variety of substituents. In certain embodiments, R_1 and R_2 may contain primary amines, secondary amines, tertiary amines, hydroxyl groups, and alkoxy groups. In certain embodiments, the polymers are amine-terminated; and in other embodiments,

the polymers are acrylate-terminated. In a particularly preferred embodiment, the groups R_1 and/or R_2 form cyclic structures with the linker A (see the Detailed Description section below). Polymers containing such cyclic moieties have the characteristic of being more soluble at lower pH. Specifically preferred polymers are

5   those that are insoluble in aqueous solutions at physiologic pH (pH 7.2-7.4) and are soluble in aqueous solutions below physiologic pH (pH < 7.2). Other preferred polymers are those that are soluble in aqueous solution at physiologic pH (pH 7.2-7.4) and below. Preferred polymers are useful in the transfection of polynucleotides into cells.

10   In another aspect, the present invention provides a method of preparing the inventive polymers. In a preferred embodiment, commercially available or readily available monomers, bis(secondary amines), primary amines, and bis(acrylate esters), are subjected to conditions which lead to the conjugate addition of the amine to the bis(acrylate ester). In a particularly preferred embodiment, each of the monomers is

15   dissolved in an organic solvent (e.g., DMSO, DMF, THF, diethyl ether, methylene chloride, hexanes, etc.), and the resulting solutions are combined and heated for a time sufficient to lead to polymerization of the monomers. In other embodiments, the polymerization is carried out in the absence of solvent. The resulting polymer may then be purified and optionally characterized using techniques known in the art.

20   In yet another aspect of the invention, the polymers are used to form nanometer-scale complexes with nucleic acids. The polynucleotide/polymer complexes may be formed by adding a solution of polynucleotide to a vortexing solution of the polymer at a desired DNA/polymer concentration. The weight to weight ratio of polynucleotide to polymer may range from 1:0.1 to 1:200, preferably from 1:10 to 1:150, more preferably

25   from 1:50 to 1:150. The amine monomer to polynucleotide phosphate ratio may be approximately 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1, 95:1, 100:1, 110:1, 120:1, 130:1, 140:1, 150:1, 160:1, 170:1, 180:1, 190:1, and 200:1. The cationic polymers condense the polynucleotide into soluble particles typically 50-500 nm in size. These polynucleotide/polymer

30   complexes may be used in the delivery of polynucleotides to cells. In a particularly preferred embodiment, these complexes are combined with pharmaceutical excipients to form pharmaceutical compositions suitable for delivery to animals including

humans. In certain embodiments, a polymer with a high molecular weight to nitrogen atom ratio (*e.g.*, polylysine, polyethyleneimine) is used to increase transfection efficiency.

In another aspect of the invention, the polymers are used to encapsulate
5   therapeutic, diagnostic, and/or prophylactic agents including polynucleotides to form microparticles. Typically these microparticles are an order of magnitude larger than the polynucleotide/polymer complexes. The microparticles range from 1 micrometer to 500 micrometers. In a particularly preferred embodiment, these microparticles allow for the delivery of labile small molecules, proteins, peptides, and/or polynucleotides to
10   an individual. The microparticles may be prepared using any of the techniques known in the art to make microparticles, such as, for example, double emulsion, phase inversion, and spray drying. In a particularly preferred embodiment, the microparticles can be used for pH-triggered delivery of the encapsulated contents due to the pH-responsive nature of the polymers (*i.e.*, being more soluble at lower pH).

15   In yet another aspect, the invention provides a system for synthesizing and screening a collection of polymers. In certain embodiments, the system takes advantage of techniques known in the art of automated liquid handling and robotics. The system of synthesizing and screening may be used with poly(beta-amino ester)s as well as other types of polymers including polyamides, polyesters, polyethers,
20   polycarbamates, polycarbonates, polyureas, polyamines, *etc.* The collection of polymers may be a collection of all one type of polymer (*e.g.*, all poly(beta-amino esters) or may be a diverse collection of polymers. Thousands of polymers may be synthesized and screened in parallel using the inventive system. In certain embodiments, the polymers are screened for properties useful in the field of gene
25   delivery, transfection, or gene therapy. Some of these properties include solubility at various pHs, ability to complex polynucleotides, ability to transfect a polynucleotide into a cell, *etc.* Certain poly(beta-amino ester)s found to be useful in transfecting cells include M17, KK89, C32, C36, JJ28, JJ32, JJ36, LL6, and D94 as described in Examples 4 and 5.

30

Definitions

The following are chemical terms used in the specification and claims:

The term *acyl* as used herein refers to a group having the general formula –C(=O)R, where R is alkyl, alkenyl, alkynyl, aryl, carbocylic, heterocyclic, or aromatic heterocyclic. An example of an acyl group is acetyl.

The term *alkyl* as used herein refers to saturated, straight- or branched-chain
5   hydrocarbon radicals derived from a hydrocarbon moiety containing between one and twenty carbon atoms by removal of a single hydrogen atom. In some embodiments, the alkyl group employed in the invention contains 1-10 carbon atoms. In another embodiment, the alkyl group employed contains 1-8 carbon atoms. In still other embodiments, the alkyl group contains 1-6 carbon atoms. In yet another embodiments,
10 the alkyl group contains 1-4 carbons. Examples of alkyl radicals include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, iso-butyl, sec-butyl, sec-pentyl, iso-pentyl, tert-butyl, n-pentyl, neopentyl, n-hexyl, sec-hexyl, n-heptyl, n-octyl, n-decyl, n-undecyl, dodecyl, and the like, which may bear one or more sustitutents.

The term *alkoxy* as used herein refers to a saturated (*i.e.*, alkyl-O-) or
15 unsaturated (*i.e.*, alkenyl-O- and alkynyl-O-) group attached to the parent molecular moiety through an oxygen atom. In certain embodiments, the alkyl group contains 1-20 aliphatic carbon atoms. In certain other embodiments, the akyl, akenyl, and alkynyl groups employed in the invention contain 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl group contains 1-6 aliphatic carbon atoms. In yet other
20 embodiments, the alkyl group contains 1-4 aliphatic carbon atoms. Examples include, but are not limited to, methoxy, ethoxy, propoxy, isopropoxy, n-butoxy, *tert*-butoxy, *i*-butoxy, *sec*-butoxy, neopentoxy, n-hexoxy, and the like.

The term *alkenyl* denotes a monovalent group derived from a hydrocarbon moiety having at least one carbon-carbon double bond by the removal of a single
25 hydrogen atom. In certain embodiments, the alkenyl group employed in the invention contains 1-20 carbon atoms. In some embodiments, the alkenyl group employed in the invention contains 1-10 carbon atoms. In another embodiment, the alkenyl group employed contains 1-8 carbon atoms. In still other embodiments, the alkenyl group contains 1-6 carbon atoms. In yet another embodiments, the alkenyl group contains 1-4
30 carbons. Alkenyl groups include, for example, ethenyl, propenyl, butenyl, 1-methyl-2-buten-1-yl, and the like.

The term *alkynyl* as used herein refers to a monovalent group derived form a

hydrocarbon having at least one carbon-carbon triple bond by the removal of a single hydrogen atom. In certain embodiments, the alkynyl group employed in the invention contains 1-20 carbon atoms. In some embodiments, the alkynyl group employed in the invention contains 1-10 carbon atoms. In another embodiment, the alkynyl group

5   employed contains 1-8 carbon atoms. In still other embodiments, the alkynyl group contains 1-6 carbon atoms. Representative alkynyl groups include, but are not limited to, ethynyl, 2-propynyl (propargyl), 1-propynyl, and the like.

        The term *alkylamino, dialkylamino,* and *trialkylamino* as used herein refers to one, two, or three, respectively, alkyl groups, as previously defined, attached to the

10   parent molecular moiety through a nitrogen atom. The term *alkylamino* refers to a group having the structure –NHR′ wherein R′ is an alkyl group, as previously defined; and the term *dialkylamino* refers to a group having the structure –NR′R″, wherein R′ and R″ are each independently selected from the group consisting of alkyl groups. The term *trialkylamino* refers to a group having the structure –NR′R″R‴, wherein R′, R″,

15   and R‴ are each independently selected from the group consisting of alkyl groups. In certain embodiments, the alkyl group contain 1-20 aliphatic carbon atoms. In certain other embodiments, the alkyl group contains 1-10 aliphatic carbon atoms. In yet other embodiments, the alkyl group contains 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl group contain 1-6 aliphatic carbon atoms. In yet other

20   embodiments, the alkyl group contain 1-4 aliphatic carbon atoms. Additionally, R′, R″, and/or R‴ taken together may optionally be –(CH$_2$)$_k$- where k is an integer from 2 to 6. Examples include, but are not limited to, methylamino, dimethylamino, ethylamino, diethylamino, diethylaminocarbonyl, methylethylamino, iso-propylamino, piperidino, trimethylamino, and propylamino.

25   The terms *alkylthioether* and *thioalkoxyl* refer to a saturated (*i.e.,* alkyl-S-) or unsaturated (*i.e.,* alkenyl-S- and alkynyl-S-) group attached to the parent molecular moiety through a sulfur atom. In certain embodiments, the alkyl group contains 1-20 aliphatic carbon atoms. In certain other embodiments, the alkyl group contains 1-10 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl

30   groups contain 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl, alkenyl, and alkynyl groups contain 1-6 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups contain 1-4 aliphatic carbon

atoms. Examples of thioalkoxyl moieties include, but are not limited to, methylthio, ethylthio, propylthio, isopropylthio, n-butylthio, and the like.

The term *aryl* as used herein refers to an unsaturated cyclic moiety comprising at least one aromatic ring. In certain embodiments, aryl group refers to a mono- or

5   bicyclic carbocyclic ring system having one or two aromatic rings including, but not limited to, phenyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl, and the like. Aryl groups can be unsubstituted or substituted with substituents selected from the group consisting of branched and unbranched alkyl, alkenyl, alkynyl, haloalkyl, alkoxy, thioalkoxy, amino, alkylamino, dialkylamino, trialkylamino, acylamino, cyano,

10   hydroxy, halo, mercapto, nitro, carboxyaldehyde, carboxy, alkoxycarbonyl, and carboxamide. In addition, substituted aryl groups include tetrafluorophenyl and pentafluorophenyl.

The term *carboxylic acid* as used herein refers to a group of formula $-CO_2H$.

The terms *halo* and *halogen* as used herein refer to an atom selected from

15   fluorine, chlorine, bromine, and iodine.

The term *heterocyclic*, as used herein, refers to an aromatic or non-aromatic, partially unsaturated or fully saturated, 3- to 10-membered ring system, which includes single rings of 3 to 8 atoms in size and bi- and tri-cyclic ring systems which may include aromatic five- or six-membered aryl or aromatic heterocyclic groups fused to a

20   non-aromatic ring. These heterocyclic rings include those having from one to three heteroatoms independently selected from oxygen, sulfur, and nitrogen, in which the nitrogen and sulfur heteroatoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. In certain embodiments, the term heterocylic refers to a non-aromatic 5-, 6-, or 7-membered ring or a polycyclic group

25   wherein at least one ring atom is a heteroatom selected from O, S, and N (wherein the nitrogen and sulfur heteroatoms may be optionally oxidized), including, but not limited to, a bi- or tri-cyclic group, comprising fused six-membered rings having between one and three heteroatoms independently selected from the oxygen, sulfur, and nitrogen, wherein (i) each 5-membered ring has 0 to 2 double bonds, each 6-membered ring has 0

30   to 2 double bonds, and each 7-membered ring has 0 to 3 double bonds, (ii) the nitrogen and sulfur heteroatoms may be optionally oxidized, (iii) the nitrogen heteroatom may optionally be quaternized, and (iv) any of the above heterocyclic rings may be fused to

an aryl or heteroaryl ring.

The term *aromatic heterocyclic*, as used herein, refers to a cyclic aromatic radical having from five to ten ring atoms of which one ring atom is selected from sulfur, oxygen, and nitrogen; zero, one, or two ring atoms are additional heteroatoms

5 independently selected from sulfur, oxygen, and nitrogen; and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms, such as, for example, pyridyl, pyrazinyl, pyrimidinyl, pyrrolyl, pyrazolyl, imidazolyl, thiazolyl, oxazolyl, isooxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinolinyl, isoquinolinyl, and the like. Aromatic heterocyclic groups can be

10 unsubstituted or substituted with substituents selected from the group consisting of branched and unbranched alkyl, alkenyl, alkynyl, haloalkyl, alkoxy, thioalkoxy, amino, alkylamino, dialkylamino, trialkylamino, acylamino, cyano, hydroxy, halo, mercapto, nitro, carboxyaldehyde, carboxy, alkoxycarbonyl, and carboxamide.

Specific heterocyclic and aromatic heterocyclic groups that may be included in

15 the compounds of the invention include: 3-methyl-4-(3-methylphenyl)piperazine, 3 methylpiperidine, 4-(bis-(4-fluorophenyl)methyl)piperazine, 4-(diphenylmethyl)piperazine, 4-(ethoxycarbonyl)piperazine, 4-(ethoxycarbonylmethyl)piperazine, 4-(phenylmethyl)piperazine, 4-(1-phenylethyl)piperazine, 4-(1,1-dimethylethoxycarbonyl)piperazine, 4-(2-(bis-(2-

20 propenyl) amino)ethyl)piperazine, 4-(2-(diethylamino)ethyl)piperazine, 4-(2-chlorophenyl)piperazine, 4-(2-cyanophenyl)piperazine, 4-(2-ethoxyphenyl)piperazine, 4-(2-ethylphenyl)piperazine, 4-(2-fluorophenyl)piperazine, 4-(2-hydroxyethyl)piperazine, 4-(2-methoxyethyl)piperazine, 4-(2-methoxyphenyl)piperazine, 4-(2-methylphenyl)piperazine, 4-(2-methylthiophenyl)

25 piperazine, 4-(2-nitrophenyl)piperazine, 4-(2-nitrophenyl)piperazine, 4-(2-phenylethyl)piperazine, 4-(2-pyridyl)piperazine, 4-(2-pyrimidinyl)piperazine, 4-(2,3-dimethylphenyl)piperazine, 4-(2,4-difluorophenyl) piperazine, 4-(2,4-dimethoxyphenyl)piperazine, 4-(2,4-dimethylphenyl)piperazine, 4-(2,5-dimethylphenyl)piperazine, 4-(2,6-dimethylphenyl)piperazine, 4-(3-

30 chlorophenyl)piperazine, 4-(3-methylphenyl)piperazine, 4-(3-trifluoromethylphenyl)piperazine, 4-(3,4-dichlorophenyl)piperazine, 4-3,4-dimethoxyphenyl)piperazine, 4-(3,4-dimethylphenyl)piperazine, 4-(3,4-

methylenedioxyphenyl)piperazine, 4-(3,4,5-trimethoxyphenyl)piperazine, 4-(3,5-
dichlorophenyl)piperazine, 4-(3,5-dimethoxyphenyl)piperazine, 4-(4-
(phenylmethoxy)phenyl)piperazine, 4-(4-(3,1-dimethylethyl)phenylmethyl)piperazine,
4-(4-chloro-3-trifluoromethylphenyl)piperazine, 4-(4-chlorophenyl)-3-

5   methylpiperazine, 4-(4-chlorophenyl)piperazine, 4-(4-chlorophenyl)piperazine, 4-(4-
chlorophenylmethyl)piperazine, 4-(4-fluorophenyl)piperazine, 4-(4-
methoxyphenyl)piperazine, 4-(4-methylphenyl)piperazine, 4-(4-nitrophenyl)piperazine,
4-(4-trifluoromethylphenyl)piperazine, 4-cyclohexylpiperazine, 4-ethylpiperazine, 4-
hydroxy-4-(4-chlorophenyl)methylpiperidine, 4-hydroxy-4-phenylpiperidine, 4-

10   hydroxypyrrolidine, 4-methylpiperazine, 4-phenylpiperazine, 4-piperidinylpiperazine,
4-(2-furanyl)carbonyl)piperazine, 4-((1,3-dioxolan-5-yl)methyl)piperazine, 6-fluoro-
1,2,3,4-tetrahydro-2-methylquinoline, 1,4-diazacylcloheptane, 2,3-dihydroindolyl, 3,3-
dimethylpiperidine, 4,4-ethylenedioxypiperidine, 1,2,3,4-tetrahydroisoquinoline,
1,2,3,4-tetrahydroquinoline, azacyclooctane, decahydroquinoline, piperazine,

15   piperidine, pyrrolidine, thiomorpholine, and triazole.

The term *carbamoyl*, as used herein, refers to an amide group of the formula
–CONH$_2$.

The term *carbonyldioxyl*, as used herein, refers to a carbonate group of the
formula –O-CO-OR.

20   The term *hydrocarbon*, as used herein, refers to any chemical group comprising
hydrogen and carbon. The hydrocarbon may be substituted or unsubstitued. The
hydrocarbon may be unsaturated, saturated, branched, unbranched, cyclic, polycyclic,
or heterocyclic. Illustrative hydrocarbons include, for example, methyl, ethyl, *n*-propyl,
iso-propyl, cyclopropyl, allyl, vinyl, *n*-butyl, *tert*-butyl, ethynyl, cyclohexyl, methoxy,

25   diethylamino, and the like. As would be known to one skilled in this art, all valencies
must be satisfied in making any substitutions.

The terms *substituted*, whether preceded by the term "optionally" or not, and
substituent, as used herein, refer to the ability, as appreciated by one skilled in this art,
to change one functional group for another functional group provided that the valency

30   of all atoms is maintained. When more than one position in any given structure may be
substituted with more than one substituent selected from a specified group, the
substituent may be either the same or different at every position. The substituents may

also be further substituted (*e.g.*, an aryl group substituent may have another substituent off it, such as another aryl group, which is further substituted with fluorine at one or more positions).

The term *thiohydroxyl* or *thiol*, as used herein, refers to a group of the formula –

5   SH.

The term *ureido*, as used herein, refers to a urea group of the formula:

–NH-CO-NH$_2$.

The following are more general terms used throughout the present specification:

10   "Animal": The term animal, as used herein, refers to humans as well as non-human animals, including, for example, mammals, birds, reptiles, amphibians, and fish. Preferably, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a primate, or a pig). An animal may be a domesticated animal. An animal may be a transgenic animal.

15   "Associated with": When two entities are "associated with" one another as described herein, they are linked by a direct or indirect covalent or non-covalent interaction. Preferably, the association is covalent. Desirable non-covalent interactions include hydrogen bonding, van der Waals interactions, hydrophobic interactions, magnetic interactions, electrostatic interactions, *etc.*

20   "Biocompatible": The term "biocompatible", as used herein is intended to describe compounds that are not toxic to cells. Compounds are "biocompatible" if their addition to cells *in vitro* results in less than or equal to 20% cell death, and their administration *in vivo* does not induce inflammation or other such adverse effects.

"Biodegradable": As used herein, "biodegradable" compounds are those that,

25   when introduced into cells, are broken down by the cellular machinery or by hydrolysis into components that the cells can either reuse or dispose of without significant toxic effect on the cells (*i.e.*, fewer than about 20 % of the cells are killed when the components are added to cells *in vitro*). The components preferably do not induce inflammation or other adverse effects *in vivo*. In certain preferred embodiments, the

30   chemical reactions relied upon to break down the biodegradable compounds are uncatalyzed.

"Effective amount": In general, the "effective amount" of an active agent or drug delivery device refers to the amount necessary to elicit the desired biological response. As will be appreciated by those of ordinary skill in this art, the effective amount of an agent or device may vary depending on such factors as the desired

5   biological endpoint, the agent to be delivered, the composition of the encapsulating matrix, the target tissue, *etc.* For example, the effective amount of microparticles containing an antigen to be delivered to immunize an individual is the amount that results in an immune response sufficient to prevent infection with an organism having the administered antigen.

10   "Peptide" or "protein": According to the present invention, a "peptide" or "protein" comprises a string of at least three amino acids linked together by peptide bonds. The terms "protein" and "peptide" may be used interchangeably. Peptide may refer to an individual peptide or a collection of peptides. Inventive peptides preferably contain only natural amino acids, although non-natural amino acids (*i.e.*, compounds

15   that do not occur in nature but that can be incorporated into a polypeptide chain) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in an inventive peptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation,

20   functionalization, or other modification, *etc.* In a preferred embodiment, the modifications of the peptide lead to a more stable peptide (*e.g.*, greater half-life *in vivo*). These modifications may include cyclization of the peptide, the incorporation of D-amino acids, *etc.* None of the modifications should substantially interfere with the desired biological activity of the peptide.

25   "Polynucleotide" or "oligonucleotide": Polynucleotide or oligonucleotide refers to a polymer of nucleotides. Typically, a polynucleotide comprises at least three nucleotides. The polymer may include natural nucleosides (*i.e.*, adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine), nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine,

30   pyrrolo-pyrimidine, 3-methyl adenosine, C5-propynylcytidine, C5-propynyluridine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine,

O(6)-methylguanine, and 2-thiocytidine), chemically modified bases, biologically modified bases (*e.g.*, methylated bases), intercalated bases, modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose), or modified phosphate groups (*e.g.*, phosphorothioates and 5'-N-phosphoramidite linkages).

5   "Small molecule": As used herein, the term "small molecule" refers to organic compounds, whether naturally-occurring or artificially created (*e.g.*, via chemical synthesis) that have relatively low molecular weight and that are not proteins, polypeptides, or nucleic acids. Typically, small molecules have a molecular weight of less than about 1500 g/mol. Also, small molecules typically have multiple carbon-

10   carbon bonds. Known naturally-occurring small molecules include, but are not limited to, penicillin, erythromycin, taxol, cyclosporin, and rapamycin. Known synthetic small molecules include, but are not limited to, ampicillin, methicillin, sulfamethoxazole, and sulfonamides.

15

Brief Description of the Drawing

Figure 1 shows the time profile for the degradation of polymers 1-3 at 37 °C at pH 5.1 and pH 7.4. Degradation is expressed as percent degradation over time based on GPC data.

20   *Figure 2* shows cytotoxicity profiles of polymers 1-3 and PEI. Viability of NIH 3T3 cells is expressed as a function of polymer concentration. The molecular weights of polymers 1, 2, and 3 were 5800, 11300, and 22500, respectively. The molecular weight of the PEI employed was 25000.

Figure 3 shows the retardation of pCMV-Luc DNA by polymer 1 in agarose gel

25   electrophoresis. Each lane corresponds to a different DNA/polymer weight ratio. The ratios are as follows: 1) 1:0 (DNA only); 2) 1:0.5; 3) 1:1; 4) 1:2; 5) 1:3; 6) 1:4; 7) 1:5; 8) 1:6; 9) 1:7; and 10) 1:8.

Figure 4 shows the average effective diameters of DNA/polymer complexes formed from pCMV-Luc plasmid and polymer 3 ($M_n = 31,000$) as a function of

30   polymer concentration.

Figure 5 shows average ζ-potentials of DNA/polymer complexes formed from pCMV-Luc plasmid and polymer 3 ($M_n = 31,000$) as a function of polymer

concentration. The numbers for each complex correspond to the complex numbers in *Figure 4*.

Figure 6 is an SEM image of rhodamine/dextran-loaded microspheres fabricated from polymer 1.

Figure 7 shows the release profiles of rhodamine/dextran from polymer 1 and PLGA microspheres at various pH values. The arrows indicate the points at which HEPES buffer (pH 7.4) was exchanged with acetate buffer (pH 5.1).

Figure 8 shows a) a representative fluorescence microscopy image of rhodamine/dextran-loaded polymer 1 microspheres suspended in HEPES buffer (pH 7.4). *Figure 8b* shows a sample of loaded polymer 1 microspheres at pH 7.4 after addition of acetate buffer (pH 5.1). The direction of diffusion of acid is from the top right to the bottom left of the image (elapsed time ≈ 5 seconds).

Figure 9 demonstrates the gel electrophoresis assay used to identify DNA-complexing polymers. Lane annotations correspond to the 70 water-soluble members of the screening library. For each polymer, assays were performed at DNA/polymer ratios of 1:5 (left well) and 1:20 (right well). Lanes marked C^* contain DNA alone (no polymer) and were used as a control.

Figure 10 shows transfection data as a function of structure for an assay employing pCMV-Luc (600 ng/well, DNA/polymer = 1:20). Light units are arbitrary and not normalized to total cell protein; experiments were performed in triplicate (error bars not shown). Black squares represent water-insoluble polymers, white squares represent water-soluble polymers that did not complex DNA in Figure 9. The right column (marked "$*$") displays values for the following control experiments: no polymer (green), PEI (red), and Lipofectamine (light blue).

Figure 11 shows a synthesis of poly(beta-amino ester)s. Poly(beta-amino ester)s may be synthesized by the conjugate addition of primary amines (equation 1) or bis(secondary amines) (equation 2) to diacrylates.

Figure 12 shows a variety of amine (A) and diacrylate (B) monomers used in the synthesis of the polymer library.

Figure 13 is a histogram of polymer transfection efficiencies. In the first screen all 2350 polymers were tested for their ability to deliver pCMV-luc DNA at N:P ratios of 40:1, 20:1, and 10:1 to COS-7 cells. Transfection efficiency is presented in ng

Luciferase per well. For reference, PEI transfection efficiency is shown. COS-7 cells readily take up naked DNA, and in our conditions produce 0.15 ± 0.05 ng per well, and the lipid reagent, Lipofectamine 2000, produces 13.5 ± 1.9 ng per well.

Figure 14. A) Optimized transfection efficiency of the top 50 polymers relative
5   to PEI and lipofectamine 2000. Polymers were tested as described in methods. In the first broad screen N:P ratios of 40:1, 20:1, and 10:1 with an n of 1 were tested. The top 93 were rescreened at six different N:P ratios = (optimal N:P form the first screen) x 1.75, 1.5, 1.25, 1.0, 0.75, and 0.5, in triplicate. Control reactions are labeled in Red, and polymers that did not bind DNA in a gel electrophoresis assay are shown in black.
10   B) DNA binding polymers as determined by agarose gel electrophoresis. The data was tabulated in the following manner: 1) fully shifted DNA is represented by (+), 2) partially shifted DNA is represented by (+/-), 3) unbound DNA is represented by (-).

Figure 15 shows the transfection of COS-7 cells using enhanced Green Fluorescent Protein plasmid. Cells were transfected at an N:P ratio of (optimal N:P
15   from the broad screen) x 1.25 with 600 ng of DNA. Regions of the well showing high transfection are shown for the following polymers: a) C36, b) D94.

Figure 16 shows how the polymer molecular weight and the chain end-group is affected by varying the amine/diacrylate ratio in the reaction mixture. Molecular weights (M_W) (relative to polystyrene standards) were determined by organic phase
20   GPC. Polymers synthesized with amine/diacrylate ratios of >1 have amine end-groups, and polymers synthesized with amine/diacrylate ratios of <1 have acrylate end-groups.

Figure 17 shows luciferase transfection results for Poly-1 as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars are not
25   shown.)

Figure 18 shows luciferase transfection results for Poly-2 as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars not shown).

Figure 19 shows the cytotoxicity of poly-1/DNA complexes as a function of
30   polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars are not shown.)

Figure 20 shows the cytotoxicity of poly-2/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars are not shown.)

5 *Figure 21* shows the relative cellular uptake level of poly-1/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars are not shown.)

Figure 22 shows the relative cellular uptake level of poly-2/DNA complexes as

10 a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains (blank squares represent conditions where cytotoxicity of the complexes prevented a reliable measurement of cellular uptake); (B) acrylate-terminated chains. (n=4, error bars not shown.)

Figure 23 shows the enhancement of transfection activity of poly-1 (amine-

15 terminated chains, M_W=13,100) based delivery vectors through the use of co-complexing agents. (A) polylysine (PLL); (B) polyethyleneimine (PEI). (n=4, error bars are not shown).

Figure 24 shows the enhancement of transfection activity of poly-2 (amine-terminated chains, MW=13,400) based delivery vectors through the use of co-

20 complexing agents. (A) poly-lysine (PLL); (B) polyethyleneimine (PEI). (n=4, error bars are not shown.)

Figure 25 is a comparison of GFP gene transfer into COS-7 cells using Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1 (w/w/w)), Poly-2/PLL (Poly-2:PLL:DNA = 15:0.4:1 (w/w/w)), Lipofectamine 2000 (μL reagent: μg DNA = 1:1), PEI (PEI:DNA

25 1:1 (w/w), N/P ~ 8), and naked DNA. Cells were seeded on 6-well plates and grown to new confluence. Cells were the incubated with complexes (5 μg DNA/well) for 1 hour, after which time complexes were removed and fresh growth media was added. Two days later GFP expression was assayed by flow cytometry. (n=3, error bars indicate one standard deviation.)

30 *Figure 26* shows GFP expression in COS-7 cells transfected using Poly-1/PLL.

Figure 27 shows GFP gene transfer into four different cell lines using Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1 (w/w/w). Cells were seeded on 6-well plates and

grown to near confluence. Cells were then incubated with complexes (5 μg DNA/well) for 1 hour, after which time complexes were removed and fresh growth media was added. Two days later GFP expression was assayed by flow cytometry. (n=5, error bars indicate one standard deviation.)

5   *Figure 28* shows the molecular weights (M_W and M_n) of polymer **G5** as a function of the mole ratio of amine:diacrylate monomers in the synthesis.

        Figure 29 shows the molecular weights (M_W and M_n) of polymer **B14** as a function of the mole ratio of amine:diacrylate monomers in the polymer synthesis.

        Figure 30 is a comparison of the function of molecular weight to

10   amine:diacrylate mole ratio for polymers **B14** and **G5**.

Detailed Description of Certain Preferred Embodiments of the Invention

        The present invention provides improved polymeric encapuslation and delivery

15   systems based on the use of β-amino ester polymers. The sytems may be used in the pharmaceutical/drug delivery arts to delivery polynucleotides, proteins, small molecules, peptides, antigen, drugs, *etc.* to a patient, tissue, organ, cell, *etc.* The present invention also provides for the preparation and screening of large collections of polymers for "hits" that are useful in the pharmaceutical and drug delivery arts.

20   The β-amino ester polymers of the present invention provide for several different uses in the drug delivery art. The polymers with their tertiary amine-containing backbones may be used to complex polynucleotides and thereby enhance the delivery of polynucleotide and prevent their degradation. The polymers may also be used in the formation of nanoparticles or microparticles containing encapsulated agents.

25   Due to the polymers' properties of being biocompatible and biodegradable, these formed particles are also biodegradable and biocompatible and may be used to provide controlled, sustained release of the encapsulated agent. These particles may also be responsive to pH changes given the fact that these polymers are typically not substantially soluble in aqueous solution at physiologic pH but are more soluble at

30   lower pH.

Polymers

The polymers of the present invention are poly(β-amino esters) containing tertiary amines in their backbones and salts thereof. The molecular weights of the inventive polymers may range from 5,000 g/mol to over 100,000 g/mol, more preferably from 4,000 g/mol to 50,000 g/mol. In a particularly preferred embodiment,

5 the inventive polymers are relatively non-cytotoxic. In another particularly preferred embodiment, the inventive polymers are biocompatible and biodegradable. In a particularly preferred embodiment, the polymers of the present invention have pK_as in the range of 5.5 to 7.5, more preferably between 6.0 and 7.0. In another particularly preferred embodiment, the polymer may be designed to have a desired pK_a between 3.0

10 and 9.0, more preferably between 5.0 and 8.0. The inventive polymers are particularly attractive for drug delivery for several reasons: 1) they contain amino groups for interacting with DNA and other negatively charged agents, for buffering the pH, for causing endosomolysis, *etc.*; 2) they contain degradable polyester linkages; 3) they can be synthesized from commercially available starting materials; and 4) they are pH

15 responsive and future generations could be engineered with a desired pK_a. In screening for transfection efficiency, the best performing polymers were hydrophobic or the diacrylate monomers were hydrophobic, and many had mono- or di-hydroxyl side chains and/or linear, bis(secondary amines) as part of their structure.

The polymers of the present invention can generally be defined by the formula

20 (I):

(I).
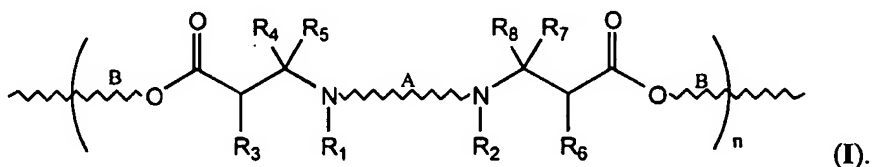
The linkers A and B are each a chain of atoms covalently linking the amino groups and ester groups, respectively. These linkers may contain carbon atoms or heteroatoms (*e.g.*, nitrogen, oxygen, sulfur, *etc.*). Typically, these linkers are 1 to 30 atoms long,

25 more preferably 1-15 atoms long. The linkers may be substituted with various substituents including, but not limited to, hydrogen atoms, alkyl, alkenyl, alkynl, amino, alkylamino, dialkylamino, trialkylamino, hydroxyl, alkoxy, halogen, aryl, heterocyclic, aromatic heterocyclic, cyano, amide, carbamoyl, carboxylic acid, ester, thioether, alkylthioether, thiol, and ureido groups. As would be appreciated by one of

30 skill in this art, each of these groups may in turn be substituted. The groups R_1, R_2, R_3,

R_4, R_5, R_6, R_7, and R_8 may be any chemical groups including, but not limited to, hydrogen atoms, alkyl, alkenyl, alkynl, amino, alkylamino, dialkylamino, trialkylamino, hydroxyl, alkoxy, halogen, aryl, heterocyclic, aromatic heterocyclic, cyano, amide, carbamoyl, carboxylic acid, ester, alkylthioether, thiol, and ureido

5   groups. In the inventive polymers, n is an integer ranging from 5 to 10,000, more preferably from 10 to 500.

In a particularly preferred embodiment, the bis(secondary amine) is a cyclic structure, and the polymer is generally represented by the formula **II**:

(II).
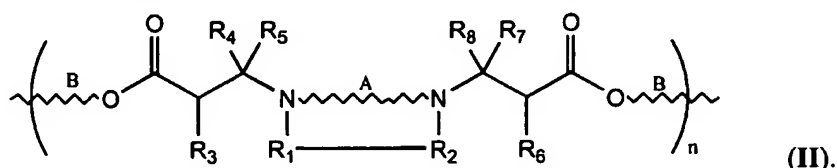
10   In this embodiment, R_1 and R_2 are directly linked together as shown in formula **II**. Examples of inventive polymers in this embodiment include, but are not limited to formulas **III** and **IV**:

(III)

15   (IV).
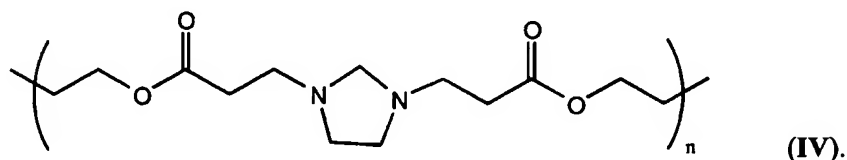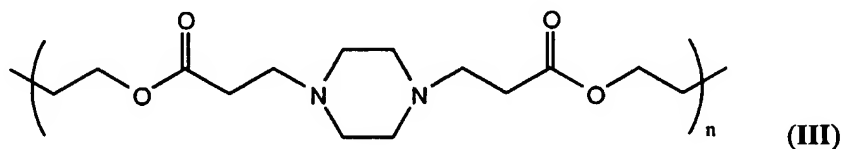
As described above in the preceding paragraph, any chemical group that satisfies the valency of each atom may be substituted for any hydrogen atom.

In another particularly preferred embodiment, the groups R_1 and/or R_2 are covalently bonded to linker A to form one or two cyclic structures. The polymers of

20   the present embodiment are generally represented by the formula **V** in which both R_1 and R_2 are bonded to linker A to form two cyclic structures:

(V).
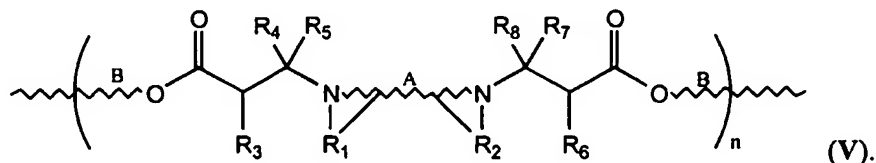
The cyclic structures may be 3-, 4-, 5-, 6-, 7-, or 8-membered rings or larger. The rings may contain heteroatoms and be unsaturated. Examples of polymers of formula **V** include formulas **VI**, **VII**, and **VIII**:

(VI)

(VII)

(VIII).
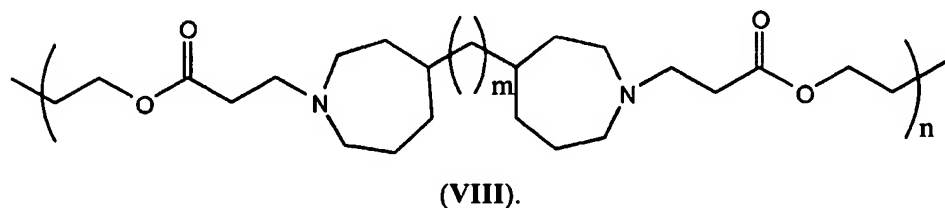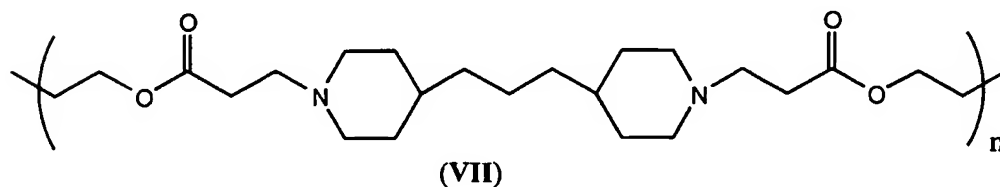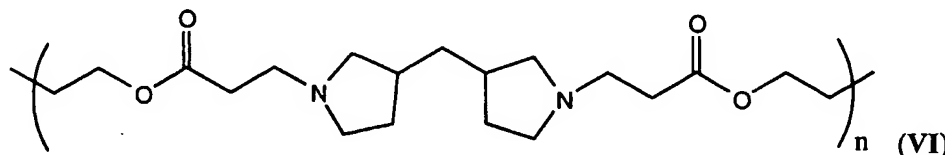
As described above, any chemical group that satisfies the valency of each atom in the molecule may be substituted for any hydrogen atom.

In another embodiment, the polymers of the present invention can generally be defined by the formula (**IX**):

(IX)
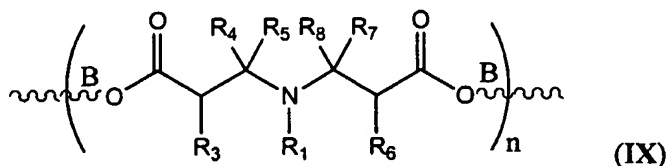
The linker B is a chain of atoms covalently linking the ester groups. The linker may contain carbon atoms or heteroatoms (*e.g.*, nitrogen, oxygen, sulfur, *etc.*). Typically, the linker is 1 to 30 atoms long, preferably 1-15 atoms long, and more preferably 2-10 atoms long. In certain embodiments, the linker B is a substituted or unsubstituted, linear or branched alkyl chain, preferably with 3-10 carbon atoms, more preferably with 3, 4, 5, 6, or 7 carbon atoms. In other embodiments, the linker B is a substituted or unsubstituted, linear or branched heteroaliphatic chain, preferably with 3-10 atoms, more preferably with 3, 4, 5, 6, or 7 atoms. In certain embodiments, the linker B is comprises of repeating units of oxygen and carbon atoms. The linker may be substituted with various substituents including, but not limited to, hydrogen atoms,

alkyl, alkenyl, alkynyl, amino, alkylamino, dialkylamino, trialkylamino, hydroxyl, alkoxy, halogen, aryl, heterocyclic, aromatic heterocyclic, cyano, amide, carbamoyl, carboxylic acid, ester, thioether, alkylthioether, thiol, acyl, acetyl, and ureido groups. As would be appreciated by one of skill in this art, each of these groups may in turn be

5   substituted. Each of R1, R3, R4, R5, R6, R7, and R8 may be independently any chemical group including, but not limited to, hydrogen atom, alkyl, alkenyl, alkynyl, amino, alkylamino, dialkylamino, trialkylamino, hydroxyl, alkoxy, halogen, aryl, heterocyclic, aromatic heterocyclic, cyano, amide, carbamoyl, carboxylic acid, ester, alkylthioether, thiol, acyl, acetyl, and ureido groups. In certain embodiments, R_1

10   includes hydroxyl groups. In other embodiments, R_1 includes amino, alkylamino, or dialkylamino groups. In the inventive polymer, n is an integer ranging from 5 to 10,000, more preferably from 10 to 500.

In certain embodiments, the polymers of the present invention are generally defined as follows:
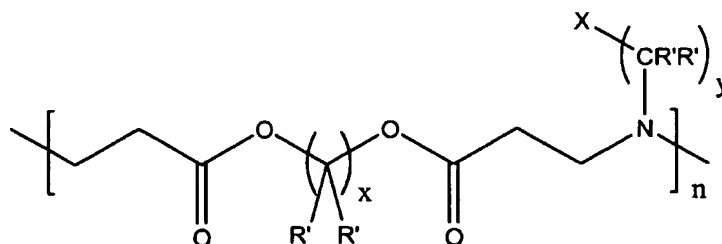
15

wherein

X is selected from the group consiting of C_1-C_6 lower alkyl, C_1-C_6 lower alkoxy, halogen, OR and NR_2; more preferably, methyl, OH, or NH_2;

R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl,

20   cyclic, heterocyclic, aryl, and heteroaryl;

each R' is independently selected from the group consisting of hydrogen, C_1-C_6 lower alkyl, C_1-C_6 lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen; preferably, R' is hydrogen, methyl, ethyl, propyl, isopropyl, butyl, methoxy, ethoxy, propoxy,

25   isopropoxy, hydroxyl, amino, fluoro, chloro, or bromo; more preferably, R' is fluoro, hydrogen, or methyl;

n is an integer between 3 and 10,000;

x is an integer between 1 and 10; preferably, x is an integer between 2 and 6;

y is an integer between 1 and 10; preferably, x is an interger between 2 and 6; and

derivatives and salts thereof.

In certain embodiments, the polymers of the present invention are generally

5   defined as follows:
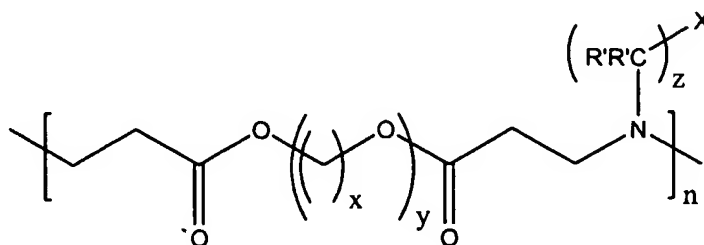
wherein

X is selected from the group consiting of C_1-C_6 lower alkyl, C_1-C_6 lower alkoxy, halogen, OR and NR_2; more preferably, methyl, OH, or NH_2;

10   R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;

each R' is independently selected from the group consisting of hydrogen, C_1-C_6 lower alkyl, C_1-C_6 lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen; preferably, R' is

15   hydrogen, methyl, ethyl, propyl, isopropyl, butyl, methoxy, ethoxy, propoxy, isopropoxy, hydroxyl, amino, fluoro, chloro, or bromo; more preferably, R' is fluoro, hydrogen, or methyl;

n is an integer between 3 and 10,000;

x is an integer between 1 and 10; preferably, x is an integer between 2 and 6;

20   y is an integer between 1 and 10; preferably, y is an interger between 2 and 6;

z is an interger between 1 and 10; preferably, z is an integer between 2 and 6; and

derivatives and salts thereof.

In another embodiment, the bis(acrylate ester) unit in the inventive polymer is

25   chosen from the following group of bis(acrylate ester) units (A'-G'):

A'

B'

C'

D'

E'

F'

G'
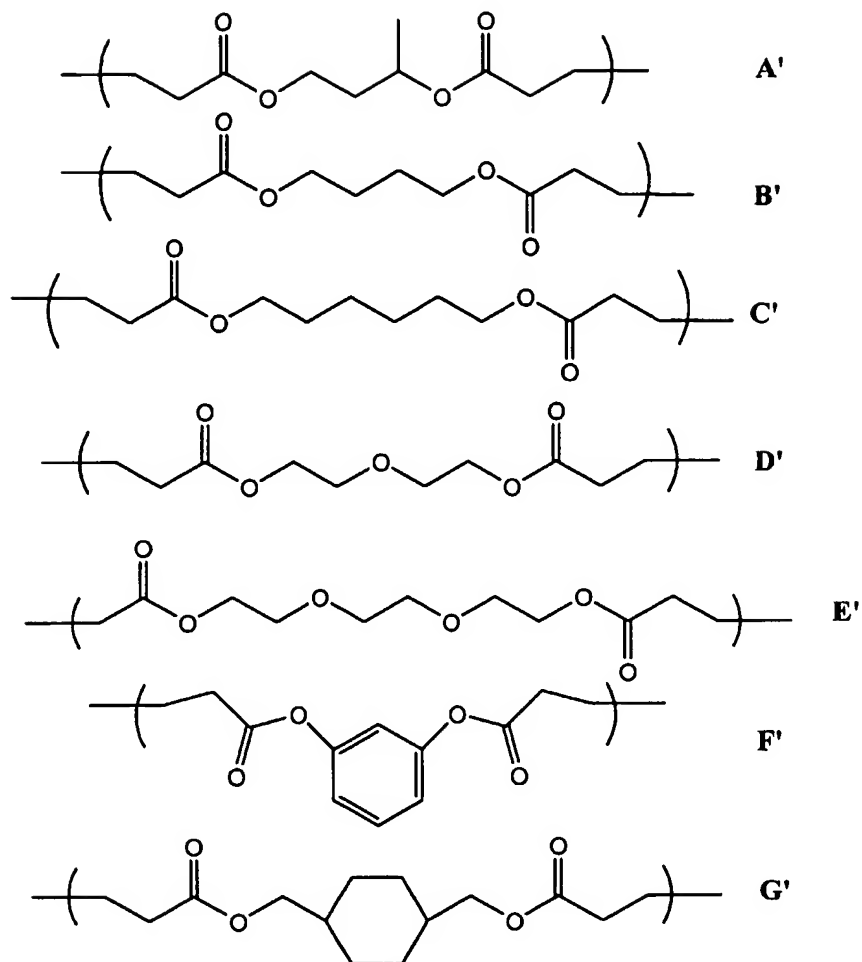
In certain embodiments, the polymer comprises the bis(acrylate ester) **G'**.

In another embodiment, the bis(acrylate ester) unit in the inventive polymer is chosen from the following group of bis(acrylate ester) units (**A-PP**):

A

B

C

D

E

F

J

K

L

M

O

P

Q

R

S

T

U

Z

AA

BB

II

JJ

KK

LL

PP

5
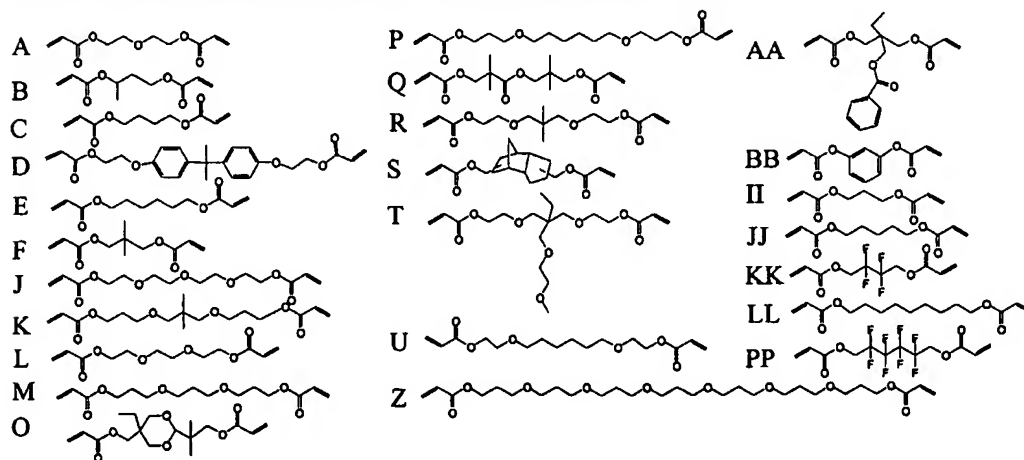
Particularly preferred bis(acrylate esters) in this group include **B, C, D, E, F, M, O, U, AA, II, JJ, KK**, and **LL**.

In another embodiment, the amine in the inventive polymer is chosen from the following group of amines (**1'-20'**):
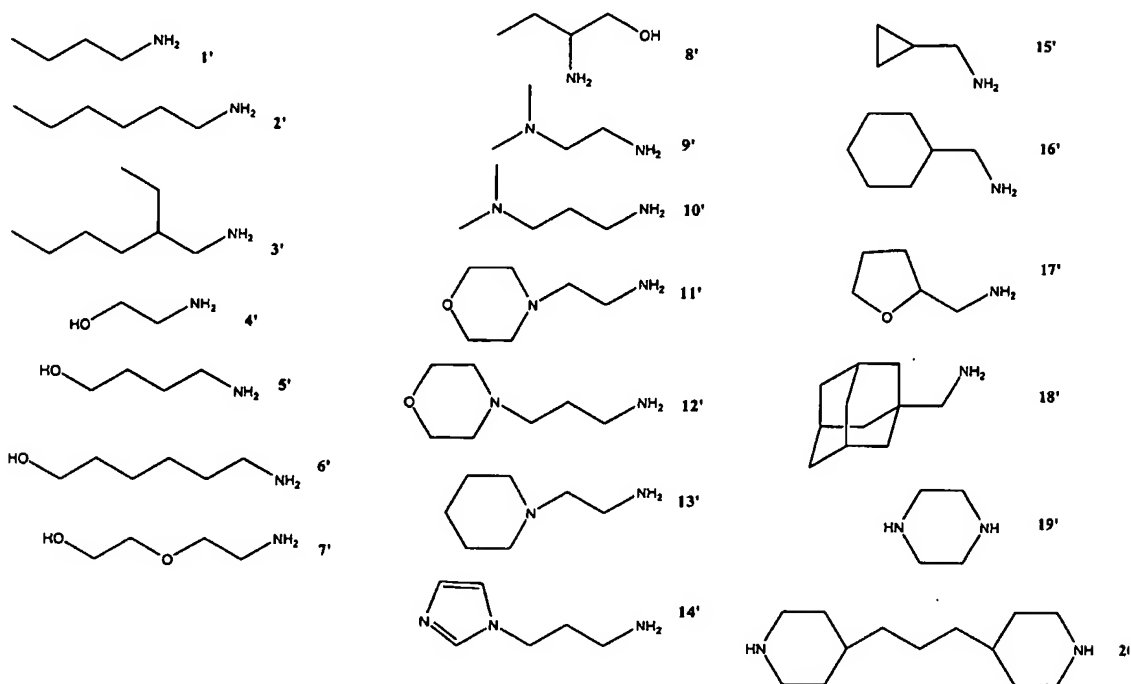
In certain embodiments, the polymer comprises the amine **5'**. In other embodiments, the polymer comprises amine **14'**.

In another embodiment, the amine in the inventive polymer is chosen from the following group of amines (**1-94**):
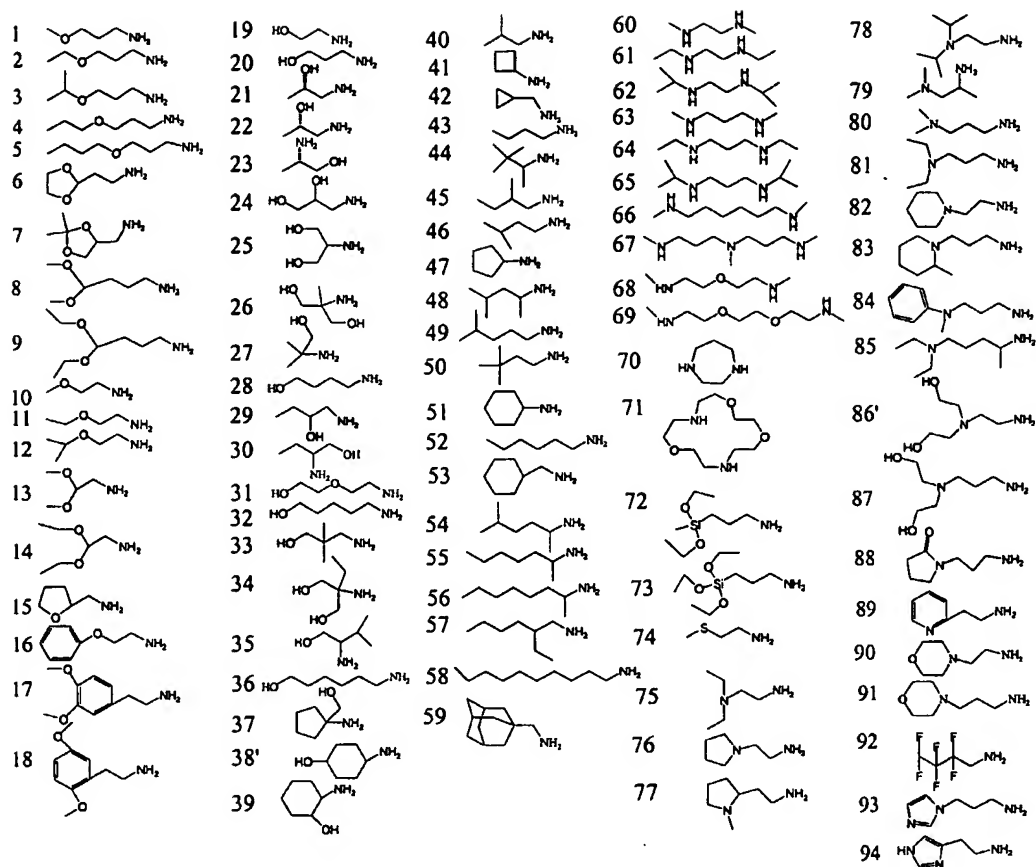
In certain embodiments, the polymers include amines **6, 8, 17, 20, 24, 25, 28, 32, 36, 60, 61, 70, 75, 80, 86, 87, 89, 93, or 94.**

Particular examples of the polymers of the present invention include:
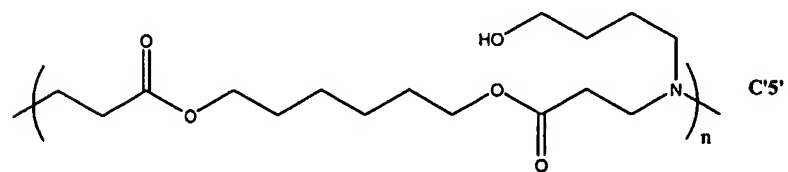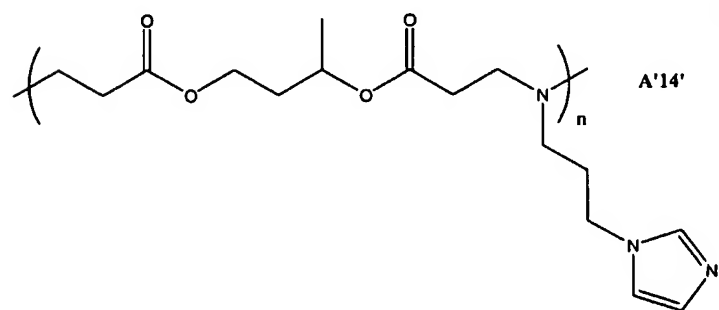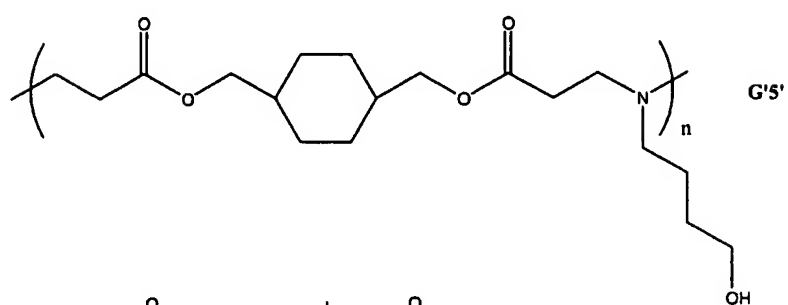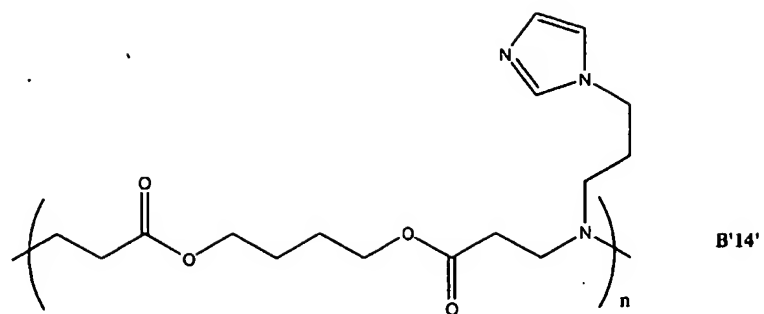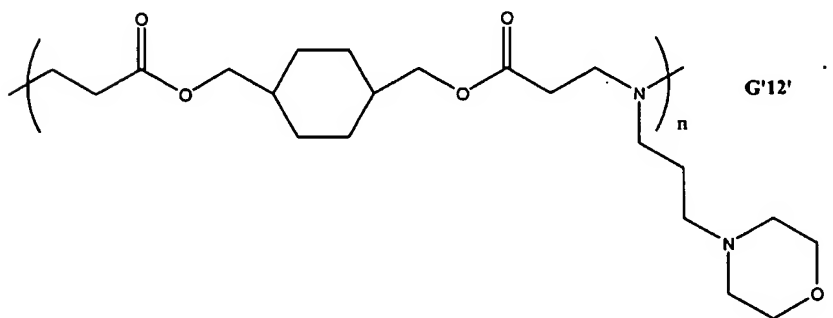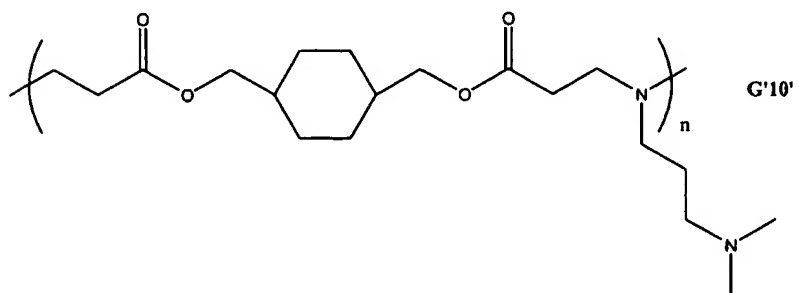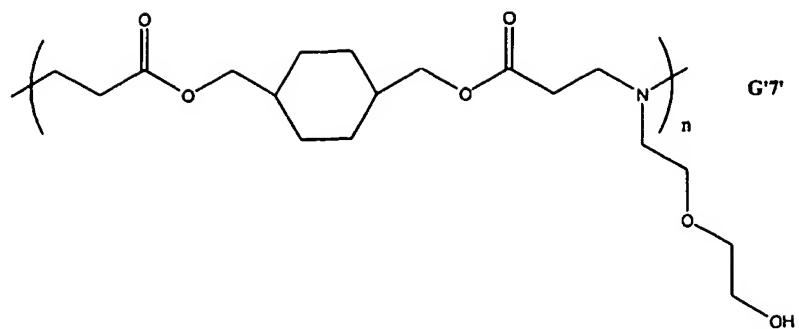
B'14'

G'5'

A'14'

C'5'

G'7'

G'10'

G'12'

Other particularly useful poly(beta-amino ester)s include:

C36

M17

KK89 and

D94.
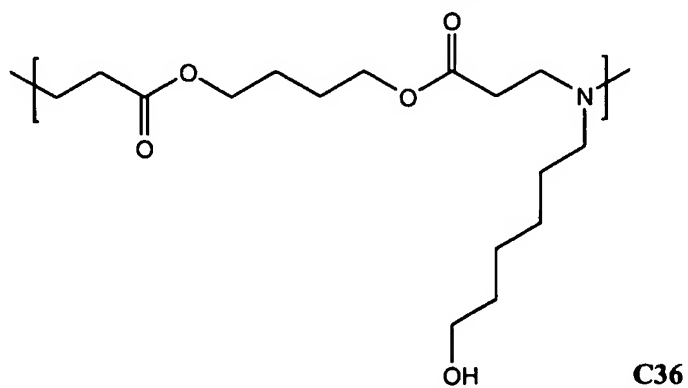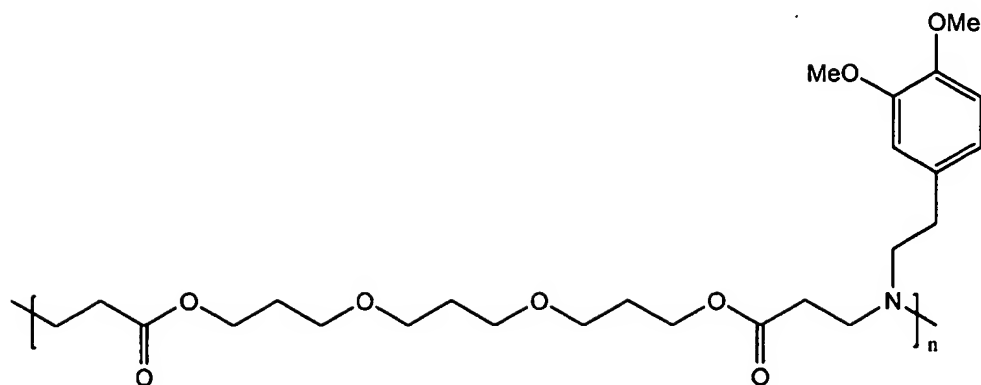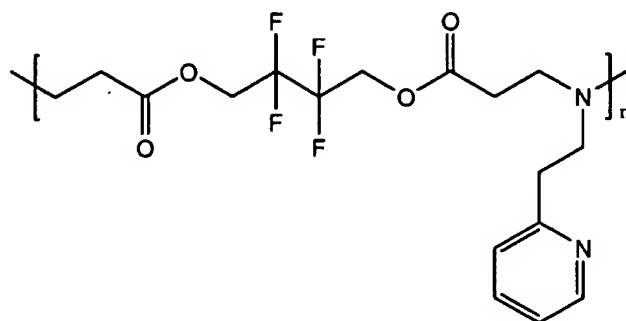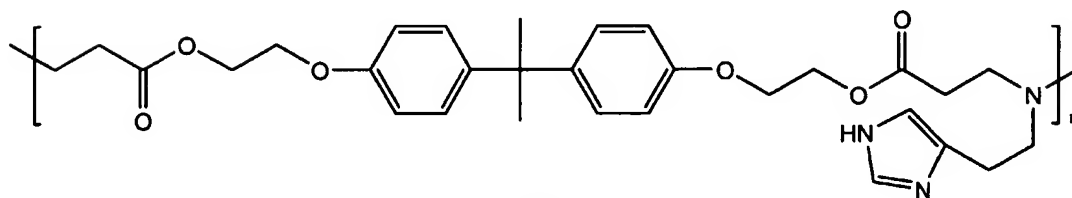
Other particularly useful poly(beta-amino esters) include C86, D60, D61, U94, F32, F28, JJ36, JJ32, LL6, LL8, U28, E28, U36, E36, U32, E32, C94, F94, JJ94, U28, JJ86, C86, U86, E86, C80, E80, JJ80, U80, D24, E24, JJ24, B17, II28, II36, II32, C20, JJ20, E20, C25, U25, D25, D70, D28, D32, D36, D93, U87, D87, C75, U75, O20, O28, C94, AA20, AA28, D86, F86, AA36, AA24, AA94, O24, AA60, A61, C32, JJ28, C28, JJ20, D94, U32, D24, C36, E28, D36, U94, E24, E32, D28, U36, E80, E36, JJ80, E94, D93, B17, M17, AA61, U93, and C25.

In a particularly preferred embodiment, one or both of the linkers A and B are polyethylene polymers. In another particularly preferred embodiment, one or both of the linkers A and B are polyethylene glycol polymers. Other biocompatible, biodegradable polymers may be used as one or both of the linkers A and B.

In certain preferred embodiments, the polymers of the present invention are amine-terminated. In other embodiments, the polymers of the present invention are acrylate-terminated. In large part, the termination unit of the polymer is determined by the ratio of amine versus acrylate in the polymer synthesis reaction. An excess of

5   amine monomer yields an amine-terminated polymer. And an excess of acrylate monomer yields an acrylate-terminated polymer.

In certain embodiments, the average molecular weight of the polymers of the present invention range from 1,000 g/mol to 50,000 g/mol, preferably from 2,000 g/mol to 40,000 g/mol, more preferably from 5,000 g/mol to 20,000 g/mol, and even more

10   preferably from 10,000 g/mol to 17,000 g/mol. Since the polymers of the present invention are prepared by a step polymerization, a broad, statistical distribution of chain lengths is typically obtained. In certain embodiments, the distribution of molecular weights in a polymer sample is narrowed by purification and isolation steps known in the art. The molecular weight of the polymer may also be varied by the synthesis of the

15   polymer, for example, as the amount of amine monomers increases, the molecular weight of the resulting polymer decreases. In other embodiments, the polymer mixture may be a blend of polymers of different molecular weights.

In another particularly preferred embodiment, the polymer of the present invention is a co-polymer wherein one of the repeating units is a poly(β-amino ester) of

20   the present invention. Other repeating units to be used in the co-polymer include, but are not limited to, polyethylene, poly(glycolide-co-lactide) (PLGA), polyglycolic acid, polymethacrylate, *etc.*

Synthesis of Polymers

25   The inventive polymers may be prepared by any method known in the art. Preferably the polymers are prepared from commercially available starting materials. In another preferred embodiment, the polymers are prepared from easily and/or inexpensively prepared starting materials.

In a particularly preferred embodiment, the inventive polymer is prepared via

30   the conjugate addition of bis(secondary amines) to bis(acrylate esters). This reaction scheme is shown below:

$$\underset{R}{H}N\!\!\sim\!\!\sim\!\!\underset{R}{N}\!\!H \;+\; \overset{O}{\underset{}{=}}\!\!\sim\!\!O\!\!\sim\!\!\sim\!\!O\overset{O}{\underset{}{=}} \longrightarrow \left(\!\!\sim\!\!O\overset{O}{\underset{}{}}\!\!\sim\!\!\underset{R}{N}\!\!\sim\!\!\sim\!\!\underset{R}{N}\!\!\sim\!\!\overset{O}{\underset{}{}}O\!\!\sim\!\!\right) \quad \textbf{(Eq 1)}$$
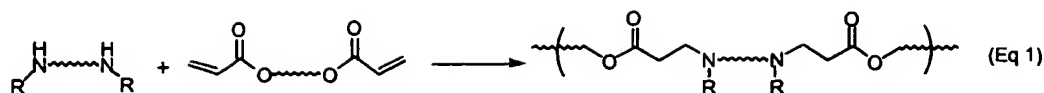
Bis(secondary amine) monomers that are useful in the present inventive method include, but are not limited to, N,N'-dimethylethylenediamine, piperazine, 2-methylpiperazine, 1,2-bis(N-ethylamino)ethylene, and 4,4'-trimethylenedipiperidine.

5   Diacrylate monomers that are useful in the present invention include, but are not limited to, 1,4-butanediol diacrylate, 1,4-butanediol dimethacrylate, 1,2-ethanediol diacrylate, 1,6-hexanediol diacrylate, 1,5-pentanediol diacrylate, 2,5-hexanediol diacrylate, and 1,3-propanediol diacrylate. Each of the monomers is dissolved in an organic solvent (*e.g.*, THF, CH_2Cl_2, MeOH, EtOH, $CHCl_3$, hexanes, toluene, benzene,

10   CCl_4, glyme, diethyl ether, DMSO, DMF, *etc.*), preferably DMSO. The resulting solutions are combined, and the reaction mixture is heated to yield the desired polymer. In other embodiments, the reaction is performed without the use of a solvent (*i.e.*, neat) thereby obviating the need for removing the solvent after the synthesis. The reaction mixture is then heated to a temperature ranging from 30 °C to 200 °C, preferably 40 °C

15   to 150 °C, more preferably 50°C to 100 °C. In a particularly preferred embodiment, the reaction mixture is heated to approximately 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, or 90 °C. In another particularly preferred embodiment, the reaction mixture is heated to approximately 75 °C. In another embodiment, the reaction mixture is heated to approximately 100 °C. The polymerization reaction may also be catalyzed. The

20   reaction time may range from hours to days depending on the polymerization reaction and the reaction conditions. As would be appreciated by one of skill in the art, heating the reaction tends to speed up the rate of reaction requiring a shorter reaction time. In certain embodiments, the polymer synthesis is carried out in DMSO at approximately 60 °C for approximately 2 days. In other emodiments, the polymer synthesis is carried

25   out without solvent at 95 °C for 8-16 hours. As would be appreciated by one of skill in this art, the molecular weight of the synthesized polymer may be determined by the reaction conditions (*e.g.*, temperature, starting materials, concentration, catalyst, solvent, time of reaction, *etc.*) used in the synthesis.

     In another particularly preferred embodiment, the inventive polymers are

30   prepared by the conjugate addition of a primary amine to a bis(acrylate ester). The use of primary amines rather than bis(secondary amines) allows for a much wider variety of

commercially available starting materials. The reaction scheme using primary amines rather than secondary amines is shown below:

$$\text{(Eq. 2)}$$
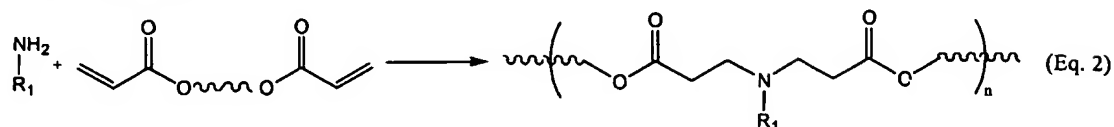
5   Primary amines useful in this method include, but are not limited to, methylamine, ethylamine, isopropylamine, aniline, substituted anilines, and ethanolamine. The bis(acrylate esters) useful in this method include, but are not limited to, 1,4-butanediol diacrylate, 1,4-butanediol dimethacrylate, 1,2-ethanediol diacrylate, 1,6-hexanediol diacrylate, 1,5-pentanediol diacrylate, 2,5-hexanediol diacrylate, and 1,3-propanediol

10   diacrylate. Each of the monomers is dissolved in an organic solvent (*e.g.*, THF, DMSO, DMF, CH_2Cl_2, MeOH, EtOH, $CHCl_3$, hexanes, CCl_4, glyme, diethyl ether, *etc.*). Organic solvents are preferred due to the susceptibility of polyesters to hydrolysis. Preferably the organic solvent used is relatively non-toxic in living systems. In certain embodiments, DMSO is used as the organic solvent because it is

15   relatively non-toxic and is frequently used as a solvent in cell culture and in storing frozen stocks of cells. Other preferred solvents include those miscible with water such as DMSO, ethanol, and methanol. The resulting solutions of monomers which are preferably at a concentration between 0.01 M and 5 M, between approximately 0.1 M and 2 M, more preferably between 0.5 M and 2 M, and most preferably between 1.3 M

20   and 1.8 M, are combined, and the reaction mixture is heated to yield the desired polymer. In certain embodiments, the reaction is run without solvent. Running the polymerization without solvent may decrease the amount of cyclization products resulting from intramolecular conjugate addition reactions. The polymerization may be run at a temperature ranging from 20 °C to 200 °C, preferably from 40 °C to 100 °C,

25   more preferably from 50 °C to 75 °C, even more preferably from 50 °C to 60 °C. In a particularly preferred embodiment, the reaction mixture is maintained at 20 °C. In another particularly preferred embodiment, the reaction mixture is heated to approximately 50 °C. In some embodiments, the reaction mixture is heated to approximately 56 °C. In yet another particularly preferred embodiment, the reaction

30   mixture is heated to approximately 75 °C. The reaction mixute may also be cooled to approximately 0 °C. The polymerization reaction may also be catalyzed such as with

an organometallic catalyste, acid, or base. In another preferred embodiment, one or more types of amine monomers and/or diacrylate monomers may be used in the polymerization reaction. For example, a combination of ethanolamine and ethylamine may be used to prepare a polymer more hydrophilic than one prepared using

5   ethylamine alone, and also more hydrophobic than one prepared using ethanolamine alone.

In preparing the polymers of the present invention, the monomers in the reaction mixture may be combined in different ratio to effect molecular weight, yield, end-termination, *etc.* of the resulting polymer. The ratio of amine monomers to

10   diacrylate monomers may range from 1.6 to 0.4, preferably from 1.4 to 0.6, more preferably from 1.2 to 0.8, even more preferably from 1.1 to 0.9. In certain embodiments, the ratio of amine monomers to diacrylate monomers is approximately 1.4, 1.3, 1.2, 1.1, 1.050, 1.025, 1.0, 0.975, 0.950, 0.900, 0.800, and 0.600. For example, combining the monomers at a ratio of 1:1 typically results in higher molecular weight

15   polymers and higher overall yields. Also, providing an excess of amine monomers (*i.e.*, amine-to-acrylate ratio > 1) results in amine-terminated chains while providing an excess of acrylate monomer (*i.e.*, amine-to-acrylate ration < 1) results in acrylate-terminated chains. The ratio of amine monomers to acrylate mononers in the polymer synthesis can affect how the polymer chains are terminated, the molecular weight of the

20   polymers produced, the distribution of molecular weights, and the extent of cross-linking.

The synthesized polymer may be purified by any technique known in the art including, but not limited to, precipitation, crystallization, extraction, chromatography, *etc.* In a particularly preferred embodiment, the polymer is purified through repeated

25   precipitations in organic solvent (*e.g.*, diethyl ether, hexane, *etc.*). In a particularly preferred embodiment, the polymer is isolated as a hydrochloride, phosphate, acetate, or other salt. Preferably the salt is pharmacuetically acceptable in certain embodiments. The resulting polymer may also be used as is without further purification and isolation; thereby making it advantageous to use a solvent compatible with the assays to be used

30   in assessing the polymers. For example, the polymers may be prepared in a non-toxic solvent such as DMSO, and the resulting solution of polymer may then be used in cell culture assays involving transfecting a nucleic acid into a cell. As would be

appreciated by one of skill in this art, the molecular weight of the synthesized polymer and the extent of cross-linking may be determined by the reaction conditions (*e.g.*, temperature, starting materials, concentration, equivalents of amine, equivalents of acrylate, order of addition, solvent, *etc.*) used in the synthesis (Odian *Principles of*

5   *Polymerization 3rd Ed.*, New York: John Wiley & Sons, 1991; Stevens *Polymer Chemistry: An Introduction 2nd Ed.*, New York: Oxford University Press, 1990; each of which is incorporated herein by reference). The extent of cross-linking of the prepared polymer may be minimized to between 1-20%, preferably between 1-10%, more preferably between 1-5%, and most preferably less than 2%. As would be

10   appreciated by those of skill in this art, amines or bis(acrylate ester)s with nucleophlic groups are more susceptible to cross-linking, and measures may need to be taken to reduce cross-linking such as lowering the temperature or changing the concetration of the starting materials in the reaction mixture. Acrylates and other moieties with unsaturation or halogens are also susceptible to radical polymerization which can lead

15   to cross-linking. The extent of radical polymerization and cross-linking may be reduced by reducing the temperature of the reaction mixture or by other means known in the art.

In one embodiment, a library of different polymers is prepared in parallel. The synthesis of a library of polymers may be carried out using any of the teachings known

20   in the art or described herein regarding the synthesis of polymers of the invention. In one embodiment, a different amine and/or bis(acrylate ester) at a particular amine-to-acrylate ratio is added to each vial in a set of vials used to prepare the library or to each well in a multi-well plate (*e.g.*, 96-well plate). In one embodiment, the monomers are diluted to between 0.1 M and 5 M, more preferably 0.5 M to 2 M, and most preferably

25   at approximately 1.6 M, in an organic solvent such as DMSO. The monomers may be combined in different ratio to effect molecular weight, yield, end-termination, *etc.* For example, combining the monomers at a ratio of 1:1 typically yields higher molecular weight polymers and higher overall yields. Providing an excess of amine monomer results in amine-terminated chains while providing an excess of acrylate monomer

30   results in acrylate-terminated chains. In some embodiments, no solvent is used in the syntheis of the polymer. The array of vials or multi-well plate is incubated at a temperature and length of time sufficient to allow polymerization of the monomers to

occur as described herein. In certain preferred embodiments, the time and temperature
are chosen to effect near complete incorporation (*e.g.*, 50% conversion, 75%
conversion, 80 % conversion, 90% conversion, 95% conversion, >95% conversion,
98% conversion, 99% conversion, or >99% conversion) of all the monomer into

5　　polymer. The polymerization reaction may be run at any temperatures ranging from 0
°C to 200 °C. In one embodiment, the reaction mixtures are incubated at approximately
45°C for approximately 5 days. In another embodiment, the reaction mixtures are
incubated at approximately 56 °C for approximately 5 days. In aother embodiment, the
reaction mixtures are incubated at approximately 100 °C for approximately 5 hours.

10　　The polymers may then be isolated and purified using techniques known in the art, or
the resulting solutions of polymers may be used without further isolation or
purification. In certain embodiments, over 1000 different polymers are prepared in
parallel. In other embodiments, over 2000 different polymers are prepared in parallel.
In still other embodiments, over 3000 different polymers are prepared in parallel. The

15　　polymers may then be screened using high-throughput techniques to identify polymers
with a desired characteristic (*e.g.*, solubility in water, solubility at different pH's,
solubility in various organic solvents, ability to bind polynucleotides, ability to bind
heparin, ability to bind small molecules, ability to form microparticles, ability to
increase tranfection efficiency, *etc.*). The polymers of the invention may be screened or

20　　used after synthesis without further precipitation, purification, or isolation of the
polymer. The use of a non-toxic solvent such as DMSO in the synthesis of the
polymers allows for the easy handling and use of the polymers after the synthesis of the
polymer. For instance, the solution of polymer in DMSO may be added to a cell
culture or other living system without a toxic effect on the cells. In certain

25　　embodiments the polymers may be screened for properties or characteristics useful in
gene therapy (*e.g.*, ability to bind polynucleotides, increase in transfection efficiency,
etc.). In other embodiments the polymers may be screened for properties or
characteristics useful in the art of tissue engineering (*e.g.*, ability to support tissue or
cell growth, ability to promote cell attachment). In certain embodiments, the polymers

30　　are synthesized and assayed using semi-automated techniques and/or robotic fluid
handling systems.

Polynucleotide Complexes

        The ability of cationic compounds to interact with negatively charged polynucleotides through electrostatic interactions is well known. Cationic polymers such as poly(lysine) have been prepared and studied for their ability to complex

5   polynucleotides. However, polymers studied to date have incorporated amines at the terminal ends of short, conformationally flexible side chains (*e.g.*, poly(lysine)) or accessible amines on the surface of spherical or globular polyamines (*e.g.*, PEI and PAMAM dendrimers). The interaction of the polymer with the polynucleotide is thought to at least partially prevent the degradation of the polynucleotide. By

10   neutralizing the charge on the backbone of the polynucleotide, the neutral or slightly-positively-charged complex is also able to more easily pass through the hydrophobic membranes (*e.g.*, cytoplasmic, lysosomal, endosomal, nuclear) of the cell. In a particularly preferred embodiment, the complex is slightly positively charged. In another particularly preferred embodiment, the complex has a postive ζ-potential, more

15   preferably the ζ-potential is between +1 and +30. In certain embodiments, agents such as polyacrylic acid (pAA), poly aspartic acid, polyglutamic acid, or poly-maleic acid may be used to prevent the serum inhibition of the polynucleotide/polymer complexes in cultured cells in media with serum (Trubetskoy *et al.* "Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery" *Gene*

20   *Therapy* 10:261-271, 2003; incorporated herein by reference).

        The poly(β-amino esters) of the present invention possess tertiary amines in the backbone of the polymer. Although these amines are more hindered, they are available to interact with a polynucleotide. Polynucleotides or derivatives thereof are contacted with the inventive polymers under conditions suitable to form polynucleotide/polymer

25   complexes. In certain embodiments, the ratio of nitrogen in the polymer (N) to phosphate in the polynucleotide is 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1, 95:1, 100:1, 110:1, or 120:1. In certain embodiments ,the polymer-to-DNA (w/w) ratio is 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1, 95:1, 100:1, 110:1,

30   120:1, 130:1, 140:1, 150:1, or 200:1. The polymer is preferably at least partially protonated so as to form a complex with the negatively charged polynucleotide. In a preferred embodiment, the polynucleotide/polymer complexes form nanoparticles that

are useful in the delivery of polynucleotides to cells. In a particularly preferred embodiment, the diameter of the nanoparticles ranges from 50-500 nm, more preferably the diameter of the nanoparticles ranges from 50-200 nm, and most preferably from 90-150 nm. The nanoparticles may be associated with a targeting agent as described

5   below.

In certain embodiments, other agents may be added to the polynucleotide:poly(beta-amino ester) complexes. In certain embodiments, a co-complexing agent is used. Co-complexing agents are known to bind polynucleotides and/or increase transfection efficiency. Co-complexing agents usually have a high

10   nitrogen density. Polylysine (PLL) and polyethylenimine (PEI) are two examples of polymeric co-complexing agents. PLL has a molecular weight to nitrogen atom ratio of 65, and PEI has a molecular weight to nitrogen atom ratio of 43. Any polymer with a molecular weight to nitrogen atom ratio in the range of 10-100, preferably 25-75, more preferably 40-70, may be useful as a co-complexing agent. The inclusion of a co-

15   complexing agent in a complex may allow one to reduce the amount of poly(beta-amino ester) in the complex. This becomes particularly important if the poly(beta-amino ester) is cytotoxic at higher concentrations. In the resulting complexes with co-complexing agents, the co-complexing agent to polynucleotide (w/w) ratio may range from 0 to 2.0, preferably from 0.1 to 1.2, more preferably from 0.1 to 0.6, and even

20   more preferably from 0.1 to 0.4.

The transfection properties of various complexes of the invention may be determined by *in vitro* transfection studies (*e.g.*, GFP transfection in cultured cells) or in animal models. In certain embodiments, the complex used for transfection is optimized for a particular cell type, polynucleotide to be delivered, poly(beta-amino

25   ester), co-complexing agent (if one is used), disease process, method of administration (*e.g.*, inhalation, oral, parenteral, IV, intrathecal, *etc.*), dosage regimen, *etc.*

Polynucleotide

The polynucleotide to be complexed or encapsulated by the inventive polymers

30   may be any nucleic acid including but not limited to RNA and DNA. The polynucleotides may be of any size or sequence, and they may be single- or double-stranded. In certain preferred embodiments, the polynucleotide is greater than 100 base

pairs long. In certain other preferred embodiments, the polynucleotide is greater than

1000 base pairs long and may be greater than 10,000 base pairs long. The

polynucleotide is preferably purified and substantially pure. Preferably, the

polynucleotide is greater than 50% pure, more preferably greater than 75% pure, and

5   most preferably greater than 95% pure. The polynucleotide may be provided by any

means known in the art. In certain preferred embodiments, the polynucleotide has been

engineered using recombinant techniques (for a more detailed description of these

techniques, please see Ausubel *et al. Current Protocols in Molecular Biology* (John

Wiley & Sons, Inc., New York, 1999); *Molecular Cloning: A Laboratory Manual*, 2nd

10   Ed., ed. by Sambrook, Fritsch, and Maniatis (Cold Spring Harbor Laboratory Press:

1989); each of which is incorporated herein by reference). The polynucleotide may

also be obtained from natural sources and purified from contaminating components

found normally in nature. The polynucleotide may also be chemically synthesized in a

laboratory. In a preferred embodiment, the polynucleotide is synthesized using

15   standard solid phase chemistry.

        The polynucleotide may be modified by chemical or biological means. In

certain preferred embodiments, these modifications lead to increased stability of the

polynucleotide. Modifications include methylation, phosphorylation, end-capping, *etc.*

        Derivatives of polynucleotides may also be used in the present invention. These

20   derivatives include modifications in the bases, sugars, and/or phosphate linkages of the

polynucleotide. Modified bases include, but are not limited to, those found in the

following nucleoside analogs: 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-

pyrimidine, 3-methyl adenosine, 5-methylcytidine, C5-bromouridine, C5-fluorouridine,

C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine,

25   7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine,

O(6)-methylguanine, and 2-thiocytidine. Modified sugars include, but are not limited

to, 2′-fluororibose, ribose, 2′-deoxyribose, 3′-azido-2′,3′-dideoxyribose, 2′,3′-

dideoxyribose, arabinose (the 2′-epimer of ribose), acyclic sugars, and hexoses. The

nucleosides may be strung together by linkages other than the phosphodiester linkage

30   found in naturally occurring DNA and RNA. Modified linkages include, but are not

limited to, phosphorothioate and 5′-N-phosphoramidite linkages. Combinations of the

various modifications may be used in a single polynucleotide. These modified

polynucleotides may be provided by any means known in the art; however, as will be appreciated by those of skill in this art, the modified polynucleotides are preferably prepared using synthetic chemistry *in vitro*.

The polynucleotides to be delivered may be in any form. For example, the
5   polynucleotide may be a circular plasmid, a linearized plasmid, a cosmid, a viral genome, a modified viral genome, an artificial chromosome, *etc.*

The polynucleotide may be of any sequence. In certain preferred embodiments, the polynucleotide encodes a protein or peptide. The encoded proteins may be enzymes, structural proteins, receptors, soluble receptors, ion channels,
10   pharmaceutically active proteins, cytokines, interleukins, antibodies, antibody fragments, antigens, coagulation factors, albumin, growth factors, hormones, insulin, *etc.* The polynucleotide may also comprise regulatory regions to control the expression of a gene. These regulatory regions may include, but are not limited to, promoters, enhancer elements, repressor elements, TATA box, ribosomal binding sites, stop site
15   for transcription, *etc.* In other particularly preferred embodiments, the polynucleotide is not intended to encode a protein. For example, the polynucleotide may be used to fix an error in the genome of the cell being transfected.

The polynucleotide may also be provided as an antisense agent or RNA interference (RNAi) (Fire *et al. Nature* 391:806-811, 1998; incorporated herein by
20   reference). Antisense therapy is meant to include, *e.g.*, administration or *in situ* provision of single- or double-stranded oligonucleotides or their derivatives which specifically hybridize, *e.g.*, bind, under cellular conditions, with cellular mRNA and/or genomic DNA, or mutants thereof, so as to inhibit expression of the encoded protein, *e.g.*, by inhibiting transcription and/or translation (Crooke "Molecular mechanisms of
25   action of antisense drugs" *Biochim. Biophys. Acta* 1489(1):31-44, 1999; Crooke "Evaluating the mechanism of action of antiproliferative antisense drugs" *Antisense Nucleic Acid Drug Dev.* 10(2):123-126, discussion 127, 2000; *Methods in Enzymology* volumes 313-314, 1999; each of which is incorporated herein by reference). The binding may be by conventional base pair complementarity, or, for example, in the case
30   of binding to DNA duplexes, through specific interactions in the major groove of the double helix (*i.e.*, triple helix formation) (Chan *et al. J. Mol. Med.* 75(4):267-282, 1997; incorporated herein by reference).

In a particularly preferred embodiment, the polynucleotide to be delivered comprises a sequence encoding an antigenic peptide or protein. Nanoparticles containing these polynucleotides can be delivered to an individual to induce an immunologic response sufficient to decrease the chance of a subsequent infection

5   and/or lessen the symptoms associated with such an infection. The polynucleotide of these vaccines may be combined with interleukins, interferon, cytokines, and adjuvants such as cholera toxin, alum, Freund's adjuvant, *etc.* A large number of adjuvant compounds are known; a useful compendium of many such compounds is prepared by the National Institutes of Health and can be found on the World Wide Web

10   (http:/www.niaid.nih.gov/daids/vaccine/pdf/compendium.pdf, incorporated herein by reference; see also Allison *Dev. Biol. Stand.* 92:3-11, 1998; Unkeless *et al. Annu. Rev. Immunol.* 6:251-281, 1998; and Phillips *et al. Vaccine* 10:151-158,1992, each of which is incorporated herein by reference).

The antigenic protein or peptides encoded by the polynucleotide may be derived

15   from such bacterial organisms as *Streptococccus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Streptococcus pyrogenes, Corynebacterium diphtheriae, Listeria monocytogenes, Bacillus anthracis, Clostridium tetani, Clostridium botulinum, Clostridium perfringens, Neisseria meningitidis, Neisseria gonorrhoeae, Streptococcus mutans, Pseudomonas aeruginosa, Salmonella typhi, Haemophilus parainfluenzae,*

20   *Bordetella pertussis, Francisella tularensis, Yersinia pestis, Vibrio cholerae, Legionella pneumophila, Mycobacterium tuberculosis, Mycobacterium leprae, Treponema pallidum, Leptospirosis interrogans, Borrelia burgdorferi, Camphylobacter jejuni,* and the like; from such viruses as smallpox, influenza A and B, respiratory syncytial virus, parainfluenza, measles, HIV, varicella-zoster, herpes simplex 1 and 2,

25   cytomegalovirus, Epstein-Barr virus, rotavirus, rhinovirus, adenovirus, papillomavirus, poliovirus, mumps, rabies, rubella, coxsackieviruses, equine encephalitis, Japanese encephalitis, yellow fever, Rift Valley fever, hepatitis A, B, C, D, and E virus, and the like; and from such fungal, protozoan, and parasitic organisms such as *Cryptococcus neoformans, Histoplasma capsulatum, Candida albicans, Candida tropicalis, Nocardia*

30   *asteroides, Rickettsia ricketsii, Rickettsia typhi, Mycoplasma pneumoniae, Chlamydial psittaci, Chlamydial trachomatis, Plasmodium falciparum, Trypanosoma brucei,*

Entamoeba histolytica, Toxoplasma gondii, Trichomonas vaginalis, Schistosoma mansoni, and the like.

Microparticles

5   The poly(β-amino esters) of the present invention may also be used to form drug delivery devices. The inventive polymers may be used to encapsulate agents including polynucleotides, small molecules, proteins, peptides, metals, organometallic compounds, *etc.* The inventive polymers have several properties that make them particularly suitable in the preparation of drug delivery devices. These include 1) the

10   ability of the polymer to complex and "protect" labile agents; 2) the ability to buffer the pH in the endosome; 3) the ability to act as a "proton sponge" and cause endosomolysis; and 4) the ability to neutralize the charge on negatively charged agents. In a preferred embodiment, the polymers are used to form microparticles containing the agent to be delivered. In a particularly preferred embodiment, the diameter of the

15   microparticles ranges from between 500 nm to 50 micrometers, more preferably from 1 micrometer to 20 micrometers, and most preferably from 1 micrometer to 10 micrometers. In another particularly preferred embodiment, the microparticles range from 1-5 micrometers. The encapsulating inventive polymer may be combined with other polymers (*e.g.*, PEG, PLGA) to form the microspheres.

20

Methods of Preparing Microparticles

      The inventive microparticles may be prepared using any method known in this art. These include, but are not limited to, spray drying, single and double emulsion solvent evaporation, solvent extraction, phase separation, simple and complex

25   coacervation, and other methods well known to those of ordinary skill in the art. Particularly preferred methods of preparing the particles are the double emulsion process and spray drying. The conditions used in preparing the microparticles may be altered to yield particles of a desired size or property (*e.g.*, hydrophobicity, hydrophilicity, external morphology, "stickiness", shape, *etc.*). The method of

30   preparing the particle and the conditions (*e.g.*, solvent, temperature, concentration, air flow rate, *etc.*) used may also depend on the agent being encapsulated and/or the composition of the polymer matrix.

Methods developed for making microparticles for delivery of encapsulated

agents are described in the literature (for example, please see Doubrow, M., Ed.,

"Microcapsules and Nanoparticles in Medicine and Pharmacy," CRC Press, Boca

Raton, 1992; Mathiowitz and Langer, *J. Controlled Release* 5:13-22, 1987; Mathiowitz

5   *et al. Reactive Polymers* 6:275-283, 1987; Mathiowitz *et al. J. Appl. Polymer Sci.*

35:755-774, 1988; each of which is incorporated herein by reference).

If the particles prepared by any of the above methods have a size range outside

of the desired range, the particles can be sized, for example, using a sieve.

10   *Agent*

The agents to be delivered by the system of the present invention may be

therapeutic, diagnostic, or prophylactic agents. Any chemical compound to be

administered to an individual may be delivered using the inventive microparticles. The

agent may be a small molecule, organometallic compound, nucleic acid, protein,

15   peptide, polynucleotide, metal, an isotopically labeled chemical compound, drug,

vaccine, immunological agent, *etc.*

In a preferred embodiment, the agents are organic compounds with

pharmaceutical activity. In another embodiment of the invention, the agent is a

clinically used drug. In a particularly preferred embodiment, the drug is an antibiotic,

20   anti-viral agent, anesthetic, steroidal agent, anti-inflammatory agent, anti-neoplastic

agent, antigen, vaccine, antibody, decongestant, antihypertensive, sedative, birth

control agent, progestational agent, anti-cholinergic, analgesic, anti-depressant, anti-

psychotic, β-adrenergic blocking agent, diuretic, cardiovascular active agent, vasoactive

agent, non-steroidal anti-inflammatory agent, nutritional agent, *etc.*

25   In a preferred embodiment of the present invention, the agent to be delivered

may be a mixture of agents. For example, a local anesthetic may be delivered in

combination with an anti-inflammatory agent such as a steroid. Local anesthetics may

also be administered with vasoactive agents such as epinephrine. To give another

example, an antibiotic may be combined with an inhibitor of the enzyme commonly

30   produced by bacteria to inactivate the antibiotic (*e.g.*, penicillin and clavulanic acid).

Diagnostic agents include gases; metals; commercially available imaging agents

used in positron emissions tomography (PET), computer assisted tomography (CAT),

single photon emission computerized tomography, x-ray, fluoroscopy, and magnetic resonance imaging (MRI); and contrast agents. Examples of suitable materials for use as contrast agents in MRI include gadolinium chelates, as well as iron, magnesium, manganese, copper, and chromium. Examples of materials useful for CAT and x-ray
5   imaging include iodine-based materials.

Prophylactic agents include, but are not limited to, antibiotics, nutritional supplements, and vaccines. Vaccines may comprise isolated proteins or peptides, inactivated organisms and viruses, dead organisms and viruses, genetically altered organisms or viruses, and cell extracts. Prophylactic agents may be combined with
10   interleukins, interferon, cytokines, and adjuvants such as cholera toxin, alum, Freund's adjuvant, *etc.* Prophylactic agents include antigens of such bacterial organisms as *Streptococccus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Streptococcus pyrogenes, Corynebacterium diphtheriae, Listeria monocytogenes, Bacillus anthracis, Clostridium tetani, Clostridium botulinum, Clostridium perfringens,*
15   *Neisseria meningitidis, Neisseria gonorrhoeae, Streptococcus mutans, Pseudomonas aeruginosa, Salmonella typhi, Haemophilus parainfluenzae, Bordetella pertussis, Francisella tularensis, Yersinia pestis, Vibrio cholerae, Legionella pneumophila, Mycobacterium tuberculosis, Mycobacterium leprae, Treponema pallidum, Leptospirosis interrogans, Borrelia burgdorferi, Camphylobacter jejuni,* and the like;
20   antigens of such viruses as smallpox, influenza A and B, respiratory syncytial virus, parainfluenza, measles, HIV, varicella-zoster, herpes simplex 1 and 2, cytomegalovirus, Epstein-Barr virus, rotavirus, rhinovirus, adenovirus, papillomavirus, poliovirus, mumps, rabies, rubella, coxsackieviruses, equine encephalitis, Japanese encephalitis, yellow fever, Rift Valley fever, hepatitis A, B, C, D, and E virus, and the like; antigens
25   of fungal, protozoan, and parasitic organisms such as *Cryptococcus neoformans, Histoplasma capsulatum, Candida albicans, Candida tropicalis, Nocardia asteroides, Rickettsia ricketsii, Rickettsia typhi, Mycoplasma pneumoniae, Chlamydial psittaci, Chlamydial trachomatis, Plasmodium falciparum, Trypanosoma brucei, Entamoeba histolytica, Toxoplasma gondii, Trichomonas vaginalis, Schistosoma mansoni,* and the
30   like. These antigens may be in the form of whole killed organisms, peptides, proteins, glycoproteins, carbohydrates, or combinations thereof.

Targeting Agents

The inventive micro- and nanoparticles may be modified to include targeting agents since it is often desirable to target a particular cell, collection of cells, or tissue. A variety of targeting agents that direct pharmaceutical compositions to particular cells

5   are known in the art (see, for example, Cotten *et al. Methods Enzym.* 217:618, 1993; incorporated herein by reference). The targeting agents may be included throughout the particle or may be only on the surface. The targeting agent may be a protein, peptide, carbohydrate, glycoprotein, lipid, small molecule, *etc.* The targeting agent may be used to target specific cells or tissues or may be used to promote endocytosis or

10   phagocytosis of the particle. Examples of targeting agents include, but are not limited to, antibodies, fragments of antibodies, low-density lipoproteins (LDLs), transferrin, asialycoproteins, gp120 envelope protein of the human immunodeficiency virus (HIV), carbohydrates, receptor ligands, sialic acid, *etc.* If the targeting agent is included throughout the particle, the targeting agent may be included in the mixture that is used

15   to form the particles. If the targeting agent is only on the surface, the targeting agent may be associated with (*i.e.*, by covalent, hydrophobic, hydrogen boding, van der Waals, or other interactions) the formed particles using standard chemical techniques.

Pharmaceutical Compositions

20   Once the microparticles or nanoparticles (polymer complexed with polynucleotide) have been prepared, they may be combined with one or more pharmaceutical excipients to form a pharmaceutical composition that is suitable to administer to animals including humans. As would be appreciated by one of skill in this art, the excipients may be chosen based on the route of administration as described

25   below, the agent being delivered, time course of delivery of the agent, *etc.*

Pharmaceutical compositions of the present invention and for use in accordance with the present invention may include a pharmaceutically acceptable excipient or carrier. As used herein, the term "pharmaceutically acceptable carrier" means a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or

30   formulation auxiliary of any type. Some examples of materials which can serve as pharmaceutically acceptable carriers are sugars such as lactose, glucose, and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as

sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered

tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes;

oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and

soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl

5   laurate; agar; detergents such as Tween 80; buffering agents such as magnesium

hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline;

Ringer's solution; ethyl alcohol; and phosphate buffer solutions, as well as other

non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate,

as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and

10   perfuming agents, preservatives and antioxidants can also be present in the

composition, according to the judgment of the formulator. The pharmaceutical

compositions of this invention can be administered to humans and/or to animals, orally,

rectally, parenterally, intracisternally, intravaginally, intranasally, intraperitoneally,

topically (as by powders, creams, ointments, or drops), bucally, or as an oral or nasal

15   spray.

Liquid dosage forms for oral administration include pharmaceutically

acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In

addition to the active ingredients (*i.e.*, microparticles, nanoparticles,

polynucleotide/polymer complexes), the liquid dosage forms may contain inert diluents

20   commonly used in the art such as, for example, water or other solvents, solubilizing

agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl

acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol,

dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor,

and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty

25   acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral

compositions can also include adjuvants such as wetting agents, emulsifying and

suspending agents, sweetening, flavoring, and perfuming agents.

Injectable preparations, for example, sterile injectable aqueous or oleaginous

suspensions may be formulated according to the known art using suitable dispersing or

30   wetting agents and suspending agents. The sterile injectable preparation may also be a

sterile injectable solution, suspension, or emulsion in a nontoxic parenterally acceptable

diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable

vehicles and solvents that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as

5   oleic acid are used in the preparation of injectables. In a particularly preferred embodiment, the particles are suspended in a carrier fluid comprising 1% (w/v) sodium carboxymethyl cellulose and 0.1% (v/v) Tween 80.

The injectable formulations can be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile

10   solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the particles with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol, or a suppository wax which are solid

15   at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the microparticles.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the particles are mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or

20   dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate,

25   e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets,

30   and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Dosage forms for topical or transdermal administration of an inventive pharmaceutical composition include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants, or patches. The particles are admixed under sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required. Ophthalmic formulation, ear drops, and eye drops are also contemplated as being within the scope of this invention.

The ointments, pastes, creams, and gels may contain, in addition to the particles of this invention, excipients such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc, and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to the particles of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants such as chlorofluorohydrocarbons.

Transdermal patches have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms can be made by dissolving or dispensing the microparticles or nanoparticles in a proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The

rate can be controlled by either providing a rate controlling membrane or by dispersing
the particles in a polymer matrix or gel.

5   These and other aspects of the present invention will be further appreciated
upon consideration of the following Examples, which are intended to illustrate certain
particular embodiments of the invention but are not intended to limit its scope, as
defined by the claims.

10   **Examples**

Example 1-Degradable Poly(β-Amino Esters): Synthesis, Characterization, and Self-Assembly with Plasmid DNA

15 **Experimental Section**

General Considerations. All manipulations involving live cells or sterile materials
were performed in a laminar flow using standard sterile technique. ^{1}H NMR (300.100
MHz) and ^{13}C NMR (75.467 MHz) spectra were recorded on a Varian Mercury
spectrometer. All chemical shift values are given in ppm and are referenced with
20 respect to residual proton or carbon signal from solvent. Organic phase gel permeation
chromatography (GPC) was performed using a Hewlett Packard 1100 Series isocratic
pump, a Rheodyne Model 7125 injector with a 100-μL injection loop, and two PL-Gel
mixed-D columns in series (5 μm, 300 x 7.5 mm, Polymer Laboratories, Amherst,
MA). THF/0.1 M piperidine was used as the eluent at a flow rate of 1.0 mL/min. Data
25 was collected using an Optilab DSP interferometric refractometer (Wyatt Technology,
Santa Barbara, CA) and processed using the TriSEC GPC software package (Viscotek
Corporation, Houston, TX). The molecular weights and polydispersities of the
polymers are reported relative to monodispersed polystyrene standards. Aqueous phase
GPC was performed by American Polymer Standards (Mentor, OH) using
30 Ultrahydrogel L and 120A columns in series (Waters Corporation, Milford, MA).
Water (1% acetic acid, 0.3 M NaCl) was used as the eluent at a flow rate of 1.0
mL/min. Data was collected using a Knauer differential refractometer and processed

using an IBM/PC GPC-PRO 3.13 software package (Viscotek Corporation, Houston, TX). The molecular weights and polydispersities of the polymers are reported relative to poly(2-vinylpyridine) standards. For cytotoxicity assays, absorbance was measured using a Dynatech Laboratories MR5000 microplate reader at 560 nm.

Materials. N,N'-dimethylethylenediamine, piperazine, and 4,4'-trimethylenedipiperidine were purchased from Aldrich Chemical Company (Milwaukee, WI). 1,4-butanediol diacrylate was purchased from Alfa Aesar Organics (Ward Hill, MA). (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Company (St. Louis, MO). Plasmid DNA (pCMV-Luc) was produced in *E. coli* (DH5α, a kind gift from Zycos, Inc., Cambridge, MA), isolated with a Qiagen kit, and purified by ethanol precipitation. NIH 3T3 cells were purchased from American Type Culture Collection (Manassas, VA) and grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium, 90%; fetal bovine serum, 10%; penicillin, 100 units/mL; streptomycin, 100 μg/mL. All other materials and solvents were used as received without further purification.

General Polymerization Procedure. In a typical experiment, 1,4-butanediol diacrylate (0.750 g, 0.714 mL, 3.78 mmol) and diamine (3.78 mmol) were weighed into two separate vials and dissolved in THF (5 mL). The solution containing the diamine was added to the diacrylate solution *via* pipette. A Teflon-coated stirbar was added, the vial was sealed with a Teflon-lined screw-cap, and the reaction was heated at 50 °C. After 48 hours, the reaction was cooled to room temperature and dripped slowly into vigorously stirring diethyl ether or hexanes. Polymer was collected and dried under vacuum prior to analysis.

Synthesis of Polymer 1. Polymer 1 was prepared according to the general procedure outlined above. ^{1}H NMR δ (CDCl$_3$, 300 MHz) 4.11 (br t, 4H), 2.75 (br t, J=7.05 Hz, 4 H), 2.53 (br s, 4H), 2.50 (br t, (obsc), J=7.20 Hz, 4H), 2.28 (br s, 6H), 1.71, (br m, 4H). ^{13}C NMR δ (CDCl$_3$, 75.47 MHz) 172.55, 64.14, 55.31, 53.39, 42.47, 32.54, 25.53.

Synthesis of Polymer 2. Polymer 2 was prepared according to the general procedure

outlined above. ^{1}H NMR δ (CDCl$_3$, 300 MHz) 4.11 (br t, 4H), 2.74 (br t, J=7.35, 4H), 2.56 (br m, 12H), 1.71 (br t, 4H). ^{13}C NMR δ (CDCl$_3$, 75.47 MHz) 172.24, 64.19, 53.55, 52.75, 32.27, 25.52.

Synthesis of Polymer 3. Polymer 3 was prepared according to the general procedure outlined above. ^{1}H NMR δ (CDCl$_3$, 300 MHz) 4.11 (br t, 4H), 3.00 (br m, 4H), 2.79 (br m, 4H), 2.65 (br m, 4H), 2.11 (br m, 4H), 1.70 (br m, 8H), 1.25 (br m, 12H). ^{13}C NMR δ (CDCl$_3$, 75.47 MHz) 172.37, 64.13, 53.89 (br), 36.74, 35.58, 32.11 (br), 25.45, 24.05.

Polymer Degradation Studies. The hydrochloride salts of polymers 1-3 were dissolved in acetate buffer (1 M, pH = 5.1) or HEPES buffer (1 M, pH = 7.4) at a concentration of 5 mg/mL (the use of millimolar concentrations of buffer resulted in substantial reduction of pH during degradation due to the production of acidic degradation products). Samples were incubated at 37 °C on a mechanical rotator, and aliquots (1 mL) were removed at appropriate time intervals. Aliquots were frozen immediately in liquid nitrogen and lyophilized. Polymer was extracted from dried buffer salts using THF/0.1 M piperidine (1 mL), and samples were analyzed directly by GPC.

Formation of DNA/Polymer Complexes and Agarose Gel Retardation Assays. DNA/polymer complexes were formed by adding 50 μL of a plasmid DNA solution (pCMV Luc, 2 μg/50 μL in water) to a gently vortexing solution of the hydrochloride salt of polymers 1-3 (50 μL in 25 mM MES, pH = 6.0, concentrations adjusted to yield desired DNA/polymer weight ratios). The samples were incubated at room temperature for 30 minutes, after which 20 μL was run on a 1% agarose gel (HEPES, 20 mM, pH = 7.2, 65V, 30 min). Samples were loaded on the gel with a loading buffer consisting of 10% Ficoll 400 (Amersham Pharmacia Biotech, Uppsala, Sweden) in HEPES (25 mM, pH = 7.2). Bromphenol blue was not included as a visual indicator in the loading buffer, since this charged dye appeared to interfere with the complexation of polymer and DNA. DNA bands were visualized under UV illumination by ethidium bromide staining.

Quasi-Elastic Laser Light Scattering (QELS) and Measurement of ζ-potentials.
QELS experiments and ζ-potential measurements were made using a ZetaPALS
dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville,

5   NY, 15 mW laser, incident beam = 676 nm). DNA/polymer complexes were formed as
described above for agarose gel retardation assays. Samples were diluted with 900 µL
of HEPES (20 mM, pH = 7.0), added to a gently vortexing sample of DNA/polymer
complex (total volume = 1 mL, pH = 7.0). Average particle sizes and ζ-potentials were
determined at 25 °C. Correlation functions were collected at a scattering angle of 90°,

10   and particle sizes were calculated using the MAS option of BIC's particle sizing
software (ver. 2.30) using the viscosity and refractive index of pure water at 25 °C.
Particle sizes are expressed as effective diameters assuming a lognormal distribution.
Average electrophoretic mobilities were measured at 25 °C using BIC PALS zeta
potential analysis software and zeta potentials were calculated using the Smoluchowsky

15   model for aqueous suspensions. Three measurements were made on each sample, and
the results are reported as average diameters and zeta potentials.

Cytotoxicity Assays. Immortalized NIH 3T3 cells were grown in 96-well plates at an
initial seeding density of 10,000 cells/well in 200 µL growth medium (90% Dulbecco's

20   modified Eagle's medium, 10% fetal bovine serum, penicillin 100 units/mL,
streptomycin 100 µg/mL). Cells were grown for 24 hours, after which the growth
medium was removed and replaced with 180 µL of serum-free medium. Appropriate
amounts of polymer were added in 20 µL aliquots. Samples were incubated at 37 °C
for 5 hours, and the metabolic activity of each well was determined using a

25   MTT/thiazolyl blue assay: to each well was added 25 µL of a 5 mg/mL solution of
MTT stock solution in sterile PBS buffer. The samples were incubated at 37 °C for 2
hours, and 100 µL of extraction buffer (20% w/v SDS in DMF/water (1:1), pH = 4.7)
was added to each well. Samples were incubated at 37 °C for 24 hours. Optical
absorbance was measured at 560 nm with a microplate reader and expressed as a

30   percent relative to control cells.

Results and Discussion

Polymer Synthesis and Characterization

The synthesis of linear poly(amido amines) containing tertiary amines in their backbones was reported by Ferruti *et al.* in 1970 *via* the addition of bifunctional amines to bisacrylamides (Anderson *Nature* 392(Suppl.):25-30, 1996; Friedman *Nature Med.*

5   2:144-147, 1996; Crystal *Science* 270:404-410, 1995; Mulligan *Science* 260:926-932, 1993; each of which is incorporated herein by reference). Linear poly(amido amines) were initially investigated as heparin and ion complexing biomaterials (Ferruti *et al. Advances in Polymer Science* 58:55-92, 1984; Ferruti *et al. Biomaterials* 15:1235-1241, 1994; Ferruti *et al. Macromol. Chem. Phys.* 200:1644-1654, 1999; Ferruti *et al.*

10   *Biomaterials* 15:1235-1241, 1994; each of which is incorporated herein by reference). Dendritic poly(amido amines) (PAMAMs) have seen increasing use in gene transfer applications due to their ability to complex DNA (Kukowska-Latallo *et al. Proc. Natl. Acad. Sci. USA* 93:4897-4902, 1996; Tang *et al. Bioconjugate Chem.* 7:703-714, 1996; Haensler *et al. Bioconjugate Chem.* 4:372-379, 1993; each of which is incorporated

15   herein by reference), and a recent report describes the application of a family of linear poly(amido amines) to cell transfection and cytotoxicity studies (Hill *et al. Biochim. Biophys. Acta* 1427:161-174, 1999; incorporated herein by reference). In contrast, analogous poly(ester amines) formed from the Michael-type addition of bifunctional amines to diacrylate esters have received less attention (Danusso *et al. Polymer* 11:88-

20   113, 1970; Ferruti *et al. Polymer* 26:1336, 1985; Ferruti *et al. Advances in Polymer Science* 58:55-92, 1984; Ferruti *et al. Biomaterials* 15:1235-1241, 1994; Ferruti *et al. Macromol. Chem. Phys.* 200:1644-1654, 1999; Ferruti *et al. Biomaterials* 15:1235-1241, 1994; Kargina *et al. Vysokomol. Soedin. Seriya A* 28:1139-1144, 1986; Rao *et al. J. Bioactive and Compatible Polymers* 14:54-63, 1999; each of which is incorporated

25   herein by reference).

The poly(amino ester) approach presents a particularly attractive basis for the development of new polymeric transfection vectors for several reasons: 1) the polymers contain the requisite amines and readily degradable linkages, 2) multiple analogs could potentially be synthesized directly from commercially available starting

30   materials, and 3) if the resulting polymers were useful as DNA condensing agents, future generations of polymer could easily be engineered to possess amine pK_a values spanning the range of physiologically relevant pH. This last point was particularly

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intriguing, because the buffering capacity of polyamines has recently been implicated as a factor influencing the escape of DNA from cell endosomes following endocytosis (Boussif *et al. Proc. Natl. Acad. Sci. USA* 92:7297-7301, 1995; Haensler *et al. Bioconjugate Chem.* 4:372-379, 1993; Behr *Chimia* 51:34-36, 1997; Demeneix *et al.*, in

5   *Artificial Self-Assembling Systems for Gene Delivery* (Felgner *et al.*, Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov *et al.*, in *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial*, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference). While the complexation of DNA with a cationic polymer is required to compact and

10   protect DNA during early events in the transfection process, DNA and polymer must ultimately decomplex in the nucleus to allow efficient transcription (Luo *et al. Nat. Biotechnol.* 18:33-37, 2000; incorporated herein by reference). In view of this requirement, degradable polycations could play an important role in "vector unpackaging" events in the nucleus (Luo *et al. Nat. Biotechnol.* 18:33-37, 2000;

15   Schaffer *et al. Biotechnol. Bioeng.* 67:598-606, 2000; Kabanov *Pharm. Sci. Technol. Today* 2:365-372, 1999; each of which is incorporated herein by reference). Finally, we hypothesized that polymers of this general structure, and the β-amino acid derivatives into which they would presumably degrade, would be significantly less toxic than poly(lysine) and PEI. As outlined above, degradable polycations (Putnam *et*

20   *al. Macromolecules* 32:3658-3662, 1999; Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; Lim *et al. J. Am. Chem. Soc.* 122:6524-6525, 2000; each of which is incorporated herein by reference) and linear polymers containing relatively hindered amines located close to the polymer backbone (Gonzalez *et al. Bioconjugate Chem.* 10:1068-1074, 1999; incorporated herein by reference) are less toxic than poly(lysine) and PEI.

25   The synthesis of polymers 1-3 *via* the addition of the bis(secondary amines), N,N'-dimethylethylenediamine, piperazine, and 4,4'-trimethylenedipiperidine, to 1,4-butanediol diacrylate was investigated (Danusso *et al. Polymer* 11:88-113, 1970; Kargina *et al. Vysokomol. Soedin. Seriya A* 28:1139-1144, 1986; each of which is incorporated herein by reference). The polymerization of these monomers proceeded in

30   THF and CH_2Cl_2 at 50 °C to yield the corresponding polymers in up to 86 % yields (Table 1). Polymers were purified through repeated precipitation into diethyl ether or hexane. Polymer 1 was isolated as a clear viscous liquid; polymers 2 and 3 were

obtained as white solids after drying under high vacuum. Alternatively, polymers 1-3 could be isolated as solid hydrochloride salts upon addition of diethyl ether/HCl to a solution of polymer in THF or CH_2Cl_2. All three polymers were soluble in organic solvents such as THF, CH_2Cl_2, $CHCl_3$, and MeOH and were also soluble in water at
5   reduced pH. Polymer 1 and the hydrochloride salts of polymers 1-3 were freely soluble in water.

Poly-1

Poly-2

Poly-3
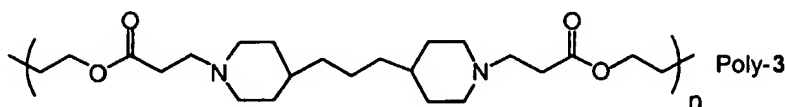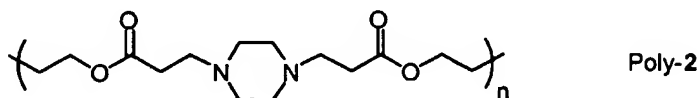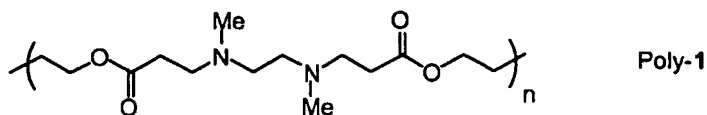
10   The molecular weights of polymers 1-3 and their corresponding hydrochloride salts were determined by both organic and aqueous phase gel permeation chromatography (GPC). Polymer molecular weights (M_n) ranged from up to 5,800 for polymer 1 to up to 32,000 for polymer 3, relative to polystyrene standards. Molecular weight distributions for these polymers were monomodal with polydispersity indices
15   (PDIs) ranging from 1.55 to 2.55. Representative molecular weight data are presented in Table 1. While the synthesis of linear poly(amido amines) is generally performed using alcohols or water as solvents (Danusso et al. Polymer 11:88-113, 1970; Ferruti et al. Polymer 26:1336, 1985; Ferruti et al. Advances in Polymer Science 58:55-92, 1984; Ferruti et al. Biomaterials 15:1235-1241, 1994; Ferruti et al. Macromol. Chem. Phys.
20   200:1644-1654, 1999; Ferruti et al. Biomaterials 15:1235-1241, 1994; each of which is incorporated herein by reference), anhydrous THF and CH_2Cl_2 were employed in the synthesis of poly(β-amino esters) to minimize hydrolysis reactions during the synthesis. The yields and molecular weights of polymers synthesized employing CH_2Cl_2 as

solvent were generally higher than those of polymers synthesized in THF (Table 1)

(Polymer 1 could not by synthesized in CH_2Cl_2. The color of the reaction solution

progressed from colorless to an intense pink color almost immediately after the

introduction of a solution of N,N'-dimethylethylenediamine in CH_2Cl_2 to a solution of

5   1,4-butanediol diacrylate in CH_2Cl_2 (see Experimental Section above). The color

progressed to light orange over the course of the reaction, and an orange polymer was

isolated after precipitation into hexane. The isolated polymer was insoluble in CH_2Cl_2,

THF, and water at reduced pH and was not structurally characterized. This problem

was not encountered for the analogous reaction in THF.).

10

Table 1. Representative Molecular Weight Data for Polymers 1-3.

Polymer	Solvent	M_n^c	PDI	Yield, %
1[a]	THF	---	---	---[d]
1[a]	CH_2Cl_2	---	---	82 %
2[a]	THF	10 000	1.77	64 %
2[a]	CH_2Cl_2	17 500	2.15	75 %
3[a]	THF	24 400	1.55	58 %
3[a]	CH_2Cl_2	30 800	2.02	70 %
1[b]	THF	5 800	2.83	55 %
2[b]	CH_2Cl_2	16 500	2.37	80 %[e]
3[b]	CH_2Cl_2	31 200	2.55	86 %[e]

[a] Conditions: [diamine] = [1,4-butanediol diacrylate] = 0.38 M, 50° C, 48 h.
[b] Conditions: [diamine] = [1,4-butanediol diacrylate] = 1.08 M, 50° C, 48 h.
[c] GPC analysis was performed in THF/0.1M piperidine and molecular weights are
15   reported versus polystyrene standards. [d] No polymer was isolated under these
conditions. [e] The reaction solution became very viscous and eventually solidified
under these conditions.

The structures of polymers 1-3 were confirmed by ^{1}H and ^{13}C NMR

20   spectroscopy. These data indicate that the polymers were formed through the conjugate

addition of the secondary amines to the acrylate moieties of 1,4-butanediol diacrylate

and not through the formation of amide linkages under our reaction conditions.

Additionally, the newly formed tertiary amines in the polymer backbones do not

participate in subsequent addition reactions with diacrylate monomer, which would

lead to branching or polymer crosslinking. This fortunate result appears to be unique to polymers of this type produced from bis(secondary amine) monomers. The synthesis of analogous polymers employing difunctional *primary* amines as monomers (such as 1,4-diaminobutane) may lead to polymer branching and the formation of insoluble

5   crosslinked polymer networks if conditions are not explicitly controlled.

In view of the juxtaposition of amines and esters within the backbones of polymers 1-3, we were initially concerned that hydrolysis might occur too rapidly for the polymers to be of practical use. For example, poly(4-hydroxy-L-proline ester) and poly[α-(4-aminobutyl)-L-glycolic acid] degrade quite rapidly near neutral pH, having

10   half lives of roughly 2 hr (Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; incorporated herein by reference) and 30 min (Lim *et al. J. Am. Chem. Soc.* 122:6524-6525, 2000; incorporated herein by reference), respectively (Such rapid degradation times did not preclude the application of these polymers to gene delivery (See references, Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; Lim *et al. J. Am. Chem.*

15   *Soc.* 122:6524-6525, 2000; each of which is incorporated herein by reference). However, extremely rapid degradation rates generally introduce additional concerns surrounding the manipulation, storage, and application of degradable polymers.). Analysis of polymers 1 and 2 by aqueous GPC using 1% acetic acid/water as eluent, however, revealed that degradation was sufficiently slow in acidic media. For example,

20   the GPC traces of polymers 1 and 2 sampled under these conditions over a period of 4 - 5 hours revealed no changes in molecular weights or polydispersities (Polymer 3 could not be analyzed by aqueous GPC.). We were also concerned that significant backbone hydrolysis might occur during the isolation of the hydrochloride salts of polymers 1-3. To prevent hydrolysis during the protonation and isolation of these polymers,

25   anhydrous solvents were employed and reactions were performed under an argon atmosphere. Analysis of the polymers before and after protonation revealed no observable hydrolysis. For example, the GPC trace of a sample of polymer 3 after precipitation from CH_2Cl_2 with 1.0 M diethyl ether/HCl (M_n = 15,300; PDI = 1.90) was virtually identical to the molecular weight of the polymer prior to protonation (M_n =

30   15,700; PDI = 1.92) and no lower molecular weight species were evident (Comparative GPC data were collected employing THF/0.1M piperidine as eluent (see Experimental Section above). The HCl salts of the polymers were insoluble in THF, but were soluble

in THF/0.1 M piperidine concomitant with the production of a fine white precipitate which was filtered prior to injection.). Solid samples of polymers 1-3 could be stored for several months without detectable decreases in molecular weight.

Polymers 1-3 were specifically designed to degrade *via* hydrolysis of the ester
5   bonds in the polymer backbones. However, an additional concern surrounding the overall stability and biocompatibility of these polymers is the potential for unwanted degradation to occur through retro-Michael reaction under physiological conditions. Because these polymers were synthesized *via* the Michael-type reaction of a secondary amine to an acrylate ester, it is possible that the polymers could undergo retro-Michael
10   reaction to regenerate free acrylate groups, particularly under acidic conditions. Acrylate esters are potential DNA-alkylating agents and are therefore suspected carcinogens (for recent examples, see: Schweikl *et al. Mutat. Res.* 438:P71-P78, 1999; Yang *et al. Carcinogenesis* 19:P1117-P1125, 1998; each of which is incorporated herein by reference). Because these polymers are expected to encounter the reduced
15   pH environment within the endosomal vesicles of cells (pH = 5.0 - 5.5) during transfection, we addressed the potential for the degradation of these polymers to occur through a retro-Michael pathway.

Under extremely acidic (pH < 3) or basic (pH > 12) conditions, polymers 1-3 degraded rapidly and exclusively to 1,4-butanediol and the anticipated bis(β-amino
20   acid) byproducts 4a-6a as determined by ^{1}H NMR spectroscopy. No spectroscopic evidence for retro-Michael addition under these conditions was found. It is worth noting that bis(β-amino acid) degradation products 4a-6a would be less likely to undergo a retro-Michael reaction, as acrylic acids are generally less activated Michael addition partners (Perlmutter, P., in *Conjugate Addition Reactions in Organic*
25   *Synthesis*, Pergamon Press, New York, 1992; incorporated herein by reference). Further degradation of compounds 4a-6a under these conditions was not observed.
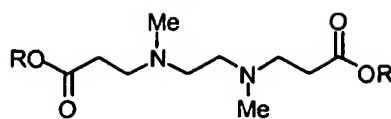
4a, R = H
4b, R = Me
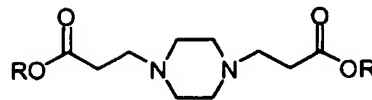
5a, R = H
5b, R = Me
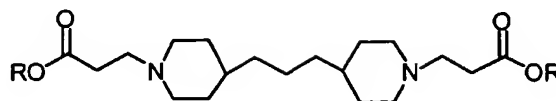
6a, R = H
6b, R = Me

The kinetics of polymer degradation were investigated under the range of conditions likely to be encountered by these polymers during transfection. Degradation

5   was monitored at 37 °C at buffered pH values of 5.1 and 7.4 in order to approximate the pH of the environments within endosomal vesicles and the cytoplasm, respectively. The hydrochloride salts of polymers 1-3 were added to the appropriate buffer, incubated at 37 °C, and aliquots were removed at appropriate times. Aliquots were frozen immediately, lyophilized, and polymer was extracted into THF/0.1 M piperidine

10   for analysis by GPC. Figure 1 shows the degradation profiles of polymers 1-3 as a function of time. The polymers degraded more slowly at pH 5.1 than at pH 7.4. Polymers 1-3 displayed similar degradation profiles at pH 5.1, each polymer having a half-life of approximately 7-8 hours. In contrast, polymers 1 and 3 were completely degraded in less than 5 hours at pH 7.4. These results are consistent with the pH-

15   degradation profiles of other amine-containing polyesters, such as poly(4-hydroxy-L-proline ester), in which pendant amine functionalities are hypothesized to act as intramolecular nucleophilic catalysts and contribute to more rapid degradation at higher pH (Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; each of which is incorporated herein by reference). While the

20   possibility of intramolecular assistance cannot be ruled out, it is less likely for polymers 1-3 because the tertiary amines in these polymers should be less nucleophilic. The degradation of polymer 2 occurred more slowly at pH 7.4 than at pH 5.1 (Figure 1). This anomalous behavior is most likely due to the incomplete solubility of polymer 2 at pH 7.4 and the resulting heterogeneous nature of the degradation milieu (Polymers 2

and 3 are not completely soluble in water at pH 7.4. While polymer 3 dissolved relatively rapidly during the degradation experiment, solid particles of polymer 2 were visible for several days.

5   Cytotoxicity Assays

Poly(lysine) and PEI have been widely studied as DNA condensing agents and transfection vectors (Luo *et al. Nat. Biotechnol.* 18:33-37, 2000; Behr *Acc. Chem. Res.* 26:274-278, 1993; Zauner *et al. Adv. Drug Del. Rev.* 30:97-113, 1998; Kabanov *et al. Bioconjugate Chem.* 6:7-20, 1995; Boussif *et al. Proc. Natl. Acad. Sci. USA* 92:7297-
10   7301, 1995; Behr *Chimia* 51:34-36, 1997; Demeneix *et al.*, in *Artificial Self-Assembling Systems for Gene Delivery* (Felgner *et al.*, Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov *et al.*, in *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial*, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference) and are the
15   standards to which new polymeric vectors are often compared (Putnam *et al. Macromolecules* 32:3658-3662, 1999; Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; Lim *et al. J. Am. Chem. Soc.* 122:6524-6525, 2000; Gonzalez *et al. Bioconjugate Chem.* 10:1068-1074, 1999; each of which is incorporated herein by reference). Unfortunately, as outlined above, these polymers are also associated with significant
20   levels of cytotoxicity and high levels of gene expression are usually realized only at a substantial cost to cell viability. To determine the toxicity profile of polymers **1-3**, a MTT/thiazolyl blue dye reduction assay using the NIH 3T3 cell line and the hydrochloride salts of polymers **1-3** was conducted as an initial indicators. The 3T3 cell line is commonly employed as a first level screening population for new
25   transfection vectors, and the MTT assay is generally used as an initial indicator of cytotoxicity, as it determines the influences of added substances on cell growth and metabolism (Hansen *et al. Immunol. Methods* 119:203-210, 1989; incorporated herein by reference).

Cells were incubated with polymer **1** ($M_n = 5\ 800$), polymer **2** ($M_n = 11\ 300$),
30   and polymer **3** ($M_n = 22\ 500$) as described in the Experimental Section. As shown in Figure 2, cells incubated with these polymers remained 100% viable relative to controls at concentrations of polymer up to 100 µg/mL. These results compare impressively to

data obtained for cell populations treated with PEI ($M_n \approx 25\ 000$), included as a positive

control for our assay as well as to facilitate comparison to this well-known transfection

agent. Fewer than 30% of cells treated with PEI remained viable at a polymer

concentration of 25 µg/mL, and cell viability was as low as 10% at higher

5   concentrations of PEI under otherwise identical conditions. An analogous MTT assay

was performed using independently synthesized bis(β-amino acid)s 4a-6a to screen the

cytotoxicity of the hydrolytic degradation products of these polymers. (Bis(β-amino

acid)s 4a-6a should either be biologically inert or possess mild or acute toxicities which

are lower than traditional polycationic transfection vectors. In either case, the

10   degradation of these materials should facilitate rapid metabolic clearance.).

Compounds 4a-6a and 1,4-butanediol did not perturb cell growth or metabolism in this

initial screening assay (data not shown). A more direct structure/function-based

comparison between polymers 1-3 and PEI cannot be made due to differences in

polymer structure and molecular weight, both of which contribute to polycation

15   toxicity. Nonetheless, the excellent cytotoxicity profiles of polymers 1-3 alone

suggested that they were interesting candidates for further study as DNA condensing

agents.

Self Assembly of Polymers 1-3 with Plasmid DNA

20   The tendency of cationic polyamines to interact electrostatically with the

polyanionic backbone of DNA in aqueous solution is well known. Provided that the

polymers are sufficiently protonated at physiological pH, and that the amines are

sterically accessible, such interactions can result in a self-assembly process in which

the positively and negatively charged polymers form well-defined conjugates (Kabanov

25   *et al.*, in *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical*

Trial, John Wiley and Sons, New York, 1998; each of which is incorporated herein by

reference). The majority of polyamines investigated as DNA-complexing agents and

transfection vectors have incorporated amines at the terminal ends of short,

conformationally flexible side chains (*e.g.*, poly(lysine) and

30   methacrylate/methacrylamide polymers) (Zauner *et al. Adv. Drug Del. Rev.* 30:97-113,

1998; Kabanov *et al. Bioconjugate Chem.* 6:7-20, 1995; van de Wetering *et al.*

Bioconjugate Chem. 10:589-597, 1999; each of which is incorporated herein by

reference), or accessible amines on the surfaces of spherical or globular polyamines (*e.g.*, PEI and PAMAM dendrimers) (Boussif *et al. Proc. Natl. Acad. Sci. USA* 92:7297-7301, 1995; Kukowska-Latallo *et al. Proc. Natl. Acad. Sci. USA* 93:4897-4902, 1996; Tang *et al. Bioconjugate Chem.* 7:703-714, 1996; Haensler *et al.*

5   *Bioconjugate Chem.* 4:372-379, 1993; each of which is incorporated herein by reference). Because polymers **1-3** contain tertiary amines, and those tertiary amines are located in a sterically crowded environment (flanked on two sides by the polymer backbones), we were initially concerned that the protonated amines might not be sufficiently able to interact intimately with DNA.

10   The ability of polymers **1-3** to complex plasmid DNA was demonstrated through an agarose gel shift assay. Agarose gel electrophoresis separates macromolecules on the basis of both charge and size. Therefore, the immobilization of DNA on an agarose gel in the presence of increasing concentrations of a polycation has been widely used as an assay to determine the point at which complete DNA charge

15   neutralization is achieved (Putnam *et al. Macromolecules* 32:3658-3662, 1999; Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; Lim *et al. J. Am. Chem. Soc.* 122:6524-6525, 2000; Gonzalez *et al. Bioconjugate Chem.* 10:1068-1074, 1999; each of which is incorporated herein by reference). As mentioned above, the hydrochloride salts of polymers **1-3** are soluble in water. However, polymers **2** and **3** are not completely

20   soluble at pH 7.2 over the full range of desired polymer concentrations. Therefore, DNA/polymer complexes were prepared in MES buffer (25 mM, pH = 6.0). DNA/polymer complexes were prepared by adding an aqueous solution of DNA to vortexing solutions of polymer in MES at desired DNA/polymer concentrations (see Experimental Section). The resulting DNA/polymer complexes remained soluble upon

25   dilution in the electrophoresis running buffer (20 mM HEPES, pH = 7.2) and data were obtained at physiological pH. As a representative example, Figure 3 depicts the migration of plasmid DNA (pCMV-Luc) on an agarose gel in the presence of increasing concentrations of polymer **1**.

As shown in Figure 3, retardation of DNA migration begins at DNA/**1** ratios as
30   low as 1:0.5 (w/w) and migration is completely retarded at DNA/polymer ratios above 1:1.0 (w/w) (DNA/polymer weight ratios rather than DNA/polymer charge ratios are reported here. Although both conventions are used in the literature, we find weight

ratios to be more practical and universal, since the overall charge on a polyamine is subject to environmental variations in pH and temperature. While DNA/polymer charge ratios are descriptive for polymers such as poly(lysine), they are less meaningful for polymers such as PEI and 1-3 which incorporate less basic amines.). Polymers **2**

5   and **3** completely inhibit the migration of plasmid DNA at DNA/polymer ratios (w/w) above 1:10 and 1:1.5, respectively (data not shown). These results vary markedly from gel retardation experiments conducted using model "monomers." Since the true monomers and the degradation products of polymers **1-3** do not adequately represent the repeat units of the polymers, we used bis(methyl ester)s **4b** - **6b** to examine the

10   extent to which the polyvalency and cooperative binding of *poly*cations **1-3** is necessary to achieve DNA immobilization. "Monomers" **4b** - **6b** did not inhibit the migration of DNA at DNA/"monomer" ratios (w/w) of up to 1:30 (data not shown).

The reasons for the less-efficient complexation employing polymer **2** in the above gel electrophoresis assays most likely results from differences in the pK_a values

15   of the amines in these polymers. The direct measurement of the pK_a values of polymers **1-3** is complicated by their degradability. However, we predict the range of pK_a values of the amines in polymers **1** and **2** to extend from approximately 4.5 and 8.0 for polymer **1**, to 3.0 and 7.0 for polymer **2**, based on comparisons to structurally related poly(β-amino amides) (The pK_a values of structurally-related poly(β-amino

20   amides) containing piperazine and dimethylethylene diamine units in their backbones have been reported. Barbucci *et al. Polymer* 21:81-85, 1980; Barbucci *et al. Polymer* 19:1329-1334, 1978; Barbucci *et al. Macromolecules* 14:1203-1209, 1981; each of which is incorporated herein by reference). As a result, polymer **2** should be protonated to a lesser extent than polymer **1** at physiological or near-neutral pH, and would

25   therefore be a less effective DNA condensing agent. The range of pK_a values for polymer **3** should be higher than the range for polymers **1** and **2** due to the increased distance between the nitrogen atoms. Accordingly, polymer **3** forms complexes with DNA at substantially reduced concentrations relative to polymer **2**.

Agarose gel retardation assays are useful in determining the extent to which

30   polycations interact with DNA. To be useful transfection agents, however, polycations must also be able to self-assemble plasmid DNA into polymer/DNA complexes small enough to enter a cell through endocytosis. For most cell types, this size requirement is

on the order of 200 nm or less (Zauner *et al. Adv. Drug Del. Rev.* 30:97-113, 1998; incorporated herein by reference), although larger particles can also be accomodated (Demeneix *et al.*, in *Artificial Self-Assembling Systems for Gene Delivery* (Felgner *et al.*, Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov

5   *et al.*, in *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial*, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference). The ability of polymers 1-3 to compact plasmid DNA into nanometer-sized structures was determined by quasi-elastic laser light scattering (QELS), and the relative surface charges of the resulting complexes were quantified through ζ-potential

10 measurements. DNA/polymer particles used for particle sizing and ζ-potential measurements were formed as described above for agarose gel electrophoresis assays and diluted in 20 mM HEPES buffer (pH = 7.0) for analysis, as described in the Experimental Section.

        Polymer 1 formed complexes with diameters ranging from 90-150 nm at

15 DNA/polymer ratios above 1:2 (w/w), and polymer 2 condensed DNA into particles on the order of 60-125 nm at DNA/polymer ratios above 1:10. These results are consistent with the data obtained from agarose gel electrophoresis experiments above. However, the particles in these experiments aggregated over a period of hours to yield larger complexes with diameters in the range of 1-2 microns. The tendency of these particles

20 to aggregate can be rationalized by the low ζ-potentials of the DNA/polymer particles observed under these conditions. For example, complexes formed from polymer 1 at DNA/polymer ratios above 1:10 had average ζ-potentials of +4.51 (±0.50) mV. The ζ-potentials of complexes formed from polymer 2 at DNA/polymer ratios above 1:20 were lower, reaching a limiting value of +1.04 (±0.57) mV. These differences correlate

25 with the estimated pK_a values for these polymers, as the surfaces of particles formed from polymer 1 would be expected to slightly more protonated than particles formed from polymer 2 at pH = 7.0.

        Polymer 3 formed complexes with diameters in the range of 50-150 nm at DNA/polymer ratios above 1:2. As a representative example, Figure 4 shows the

30 average effective diameters of particles formed with polymer 3 as a function of polymer concentration. The diameters of the particles varied within the above range from experiment to experiment under otherwise identical conditions, possibly due to

subtle differences during the stirring or addition of DNA solutions during complex

formation (The order of addition of polymer and DNA solutions had considerable

impact on the nature of the resulting DNA/polymer complexes. In order to form small

particles, for example, it was necessary to add the DNA solution to a vortexing solution

5   of polymer. For cases in which polymer solutions were added to DNA, only large

micron-sized aggregates were observed. Thus, it is possible that subtle differences in

stirring or rate of addition could be responsible for variation in particle size). The ζ-

potentials for complexes formed from polymer 3 were on the order of +10 to +15 mV

at DNA/polymer ratios above 1:1, and the complexes did not aggregate extensively

10   over an 18 hour period (pH = 7, 25 °C.) The positive ζ-potentials of these complexes

may be significant beyond the context of particle stability, as net positive charges on

particle surfaces may play a role in triggering endocytosis (Kabanov *et al. Bioconjugate

Chem.* 6:7-20, 1995; Lim *et al. J. Am. Chem. Soc.* 122:6524-6525, 2000; Behr *Chimia*

51:34-36, 1997; Demeneix *et al.*, in *Artificial Self-Assembling Systems for Gene

15   *Delivery* (Felgner *et al.*, Eds.), American Chemical Society, Washington, D.C., 1996,

pp. 146-151; Kabanov *et al.*, in *Self-Assembling Complexes for Gene Delivery: From

Laboratory to Clinical Trial*, John Wiley and Sons, New York, 1998; each of which is

incorporated herein by reference).

Particles formed from polymer 3 were also relatively stable at 37 °C. For

20   example, a sample of DNA/3 (DNA/3 = 1:5, average diameter = 83 nm) was incubated

at 37 °C for 4 hours. After 4 hours, a bimodal distribution was observed consisting of a

fraction averaging 78 nm (>98% by number, 70% by volume) and a fraction of larger

aggregates with average diameters of approximately 2.6 microns. These results suggest

that the degradation of complexes formed from polymer 3 occurred more slowly than

25   the degradation of polymer in solution, or that partial degradation did not significantly

affect the stability of the particles. The apparently increased stability of DNA/polymer

complexes formed from degradable polycations relative to the degradation of the

polymers in solution has also been observed for DNA/polymer complexes formed from

poly(4-hydroxy-L-proline ester) (Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999;

30   incorporated herein by reference).

The particle size and ζ-potential data in Figures 4 and 5 are consistent with

models of DNA condensation observed with other polycations (Kabanov *et al.*

Bioconjugate Chem. 6:7-20, 1995; Putnam *et al. Macromolecules* 32:3658-3662, 1999;
Lim *et al. J. Am. Chem. Soc.* 121:5633-5639, 1999; Lim *et al. J. Am. Chem. Soc.*
122:6524-6525, 2000; Gonzalez *et al. Bioconjugate Chem.* 10:1068-1074, 1999; each
of which is incorporated herein by reference). DNA is compacted into small negatively

5   charged particles at very low polymer concentrations and particle sizes increase with
increasing polymer concentration (Accurate light scattering data could not be obtained
for DNA alone or for DNA/polymer associated species at DNA/polymer ratios lower
than 1:0.5, since flexible, uncondensed DNA does not scatter light as extensively as
compacted DNA (Kabanov *et al.*, in *Self-Assembling Complexes for Gene Delivery:*

10   *From Laboratory to Clinical Trial*, John Wiley and Sons, New York, 1998;
incorporated herein by reference).). Complexes reach a maximum diameter as charge
neutrality is achieved and aggregation occurs. Particle sizes decrease sharply at
DNA/polymer concentrations above charge neutrality up to ratios at which additional
polymer does not contribute to a reduction in particle diameter. This model is

15   confirmed by ζ-potential measurements made on complexes formed from these
polymers. As shown in Figure 5, the ζ-potentials of polymer/DNA particles formed
from polymer **3** were negative at low polymer concentrations and charge neutrality was
achieved near DNA/polymer ratios of 1:0.75, resulting in extensive aggregation. The ζ-
potentials of the particles approached a limiting value ranging from +10 to +15 mV at

20   DNA/polymer ratios above 1:2.

The average diameters of the complexes described above fall within the general
size requirements for cellular endocytosis. We have initiated transfection experiments
employing the NIH 3T3 cell line and the luciferase reporter gene (pCMV-Luc). Thus
far, polymers **1** and **2** have shown no transfection activity in initial screening assays.

25   By contrast, polymer **3** has demonstrated transfection efficiencies exceeding those of
PEI under certain conditions. Transfection experiments were performed according to
the following general protocol: Cells were grown in 6-well plates at an initial seeding
density of 100,000 cells/well in 2 mL of growth medium. Cells were grown for 24
hours after which the growth medium was removed and replaced with 2 mL of serum-

30   free medium. DNA/polymer complexes were formed as described in the Experimental
Section (2 μg DNA, DNA/3 = 1:2 (w/w), 100 μL in MES (pH = 6.0)] and added to each
well. DNA/PEI complexes were formed at a weight ratio of 1:0.75, a ratio generally

found in our laboratory to be optimal for PEI transfections. Transfections were carried out in serum-free medium for 4 hours, after which medium was replaced with growth medium for 20 additional hours. Relative transfection efficiencies were determined using luciferase (Promega) and cell protein assay (Pierce) kits. Results are expressed as

5   relative light units (RLU) per mg of total cell protein: for complexes of polymer **3**, 1.07 ($\pm$.43) x 10^6 RLU/mg; for PEI complexes, 8.07 ($\pm$.16) x 10^5 RLU/mg). No luciferase expression was detected for control experiments employing naked DNA or performed in the absence of DNA. These transfection data are the results of initial screening experiments. These data suggest that polymers of this general structure hold promise as

10   synthetic vectors for gene delivery and are interesting candidates for further study. The relative efficacy of polymer **3** relative to PEI is interesting, as our initial screening experiments were performed in the absence of chloroquine and polymer **3** does not currently incorporate an obvious means of facilitating endosomal escape. It should be noted, however, that the pK_a values of the amines in these polymers can be "tuned" to

15   fall more directly within the range of physiologically relevant pH using this modular synthetic approach. Therefore, it will be possible to further engineer the "proton sponge" character (Behr *Chimia* 51:34-36, 1997; Demeneix *et al.*, in *Artificial Self-Assembling Systems for Gene Delivery* (Felgner *et al.*, Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov *et al.*, in *Self-Assembling*

20   *Complexes for Gene Delivery: From Laboratory to Clinical Trial*, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference) of these polymers, and thus enhance their transfection efficacies, directly through the incorporation of or copolymerization with different diamine monomers.

25   **Summary**

A general strategy for the preparation of new degradable polymeric DNA transfection vectors is reported. Poly(β-amino esters) 1-3 were synthesized *via* the conjugate addition of N,N'-dimethylethylenediamine, piperazine, and 4,4'-trimethylenedipiperidine to 1,4-butanediol diacrylate. The amines in the bis(secondary

30   amine) monomers actively participate in bond-forming processes during polymerization, obviating the need for amine protection/deprotection processes which characterize the synthesis of other poly(amino esters). Accordingly, this approach

enables the generation of a variety of structurally diverse polyesters containing tertiary amines in their backbones in a single step from commercially available staring materials. Polymers 1-3 degraded hydrolytically in acidic and alkaline media to yield 1,4-butanediol and β-amino acids **4a-6a** and the degradation kinetics were investigated

5   at pH 5.1 and 7.4. The polymers degraded more rapidly at pH 7.4 than at pH 5.1, consistent with the pH/degradation profiles reported for other poly(amino esters). An initial screening assay designed to determine the effects of polymers 1-3 on cell growth and metabolism suggested that these polymers and their hydrolytic degradation products were non-cytotoxic relative to PEI, a non-degradable cationic polymer

10   conventionally employed as a transfection vector.

Polymers 1-3 interacted electrostatically with plasmid DNA at physiological pH, initiating self-assembly processes that resulted in nanometer-scale DNA/polymer complexes. Agarose gel electrophoresis, quasi-elastic dynamic light scattering (QELS), and zeta potential measurements were used to determine the extent of the interactions

15   between the oppositely charged polyelectrolytes. All three polymers were found to condense DNA into soluble DNA/polymer particles on the order of 50-200 nm. Particles formed from polymers 1 and 2 aggregated extensively, while particles formed from polymer 3 exhibited positive ζ-potentials (*e.g.*, +10 to +15 mV) and did not aggregate for up to 18 hours. The nanometer-sized dimensions and reduced

20   cytotoxicities of these DNA/polymer complexes suggest that polymers 1-3 may be useful as degradable polymeric gene transfection vectors. A thorough understanding of structure/activity relationships existing for this class of polymer will expedite the design of safer polymer-based alternatives to viral transfection vectors for gene therapy.

25

Example 2 - Rapid, pH-Triggered Release from Biodegradable Poly(β-Amino Ester) Microspheres within the Ranger of Intracellular pH

Experimental Section

30   Fabrication of microspheres. The optimized procedure for the fabrication of microspheres was conducted in the following general manner: An aqueous solution of rhodamine-conjugated dextran (200 μL of a 10 μg/μL solution, $M_n \approx 70$ kD) was

suspended in a solution of poly-1 in CH_2Cl_2 (200 mg of poly-1 in 4 mL CH_2Cl_2, $M_n \approx$
10 kD), and the mixture was sonicated for 10 seconds to form a primary emulsion. The
cloudy pink emulsion was added directly to a rapidly homogenized (5,000 rpm)
solution of poly(vinyl alcohol) [50 mL, 1% PVA (w/w)] to form the secondary
5   emulsion. The secondary emulsion was homogenized for 30 seconds before adding it
to a second aqueous PVA solution [100 mL, 0.5% PVA (w/w)]. Direct analysis of the
microsphere suspension using a Coulter microparticle analyzer revealed a mean particle
size of approximately 5 micrometers. The secondary emulsion was stirred for 2.5 hours
at room temperature, transferred to a cold room (4 °C), and stirred for an additional 30
10   minutes. Microspheres were isolated at 4 °C *via* centrifugation, resuspended in cold
water, and centrifuged again to remove excess PVA. The spheres were resuspended in
water (15 mL) and lyophilized to yield a pink, fluffy powder. Characterization of the
lyophilized microspheres was performed by optical, fluorescence, and scanning
electron microscopies as described. Zeta potential was determined using a Brookhaven
15   Instruments ZetaPALS analyzer.

Discussion

Microparticles formed from biodegradable polymers are attractive for use as
delivery devices, and a variety of polymer-based microspheres have been employed for
20   the sustained release of therapeutic compounds (Anderson *Nature* 392(Suppl.):25-30,
1996; Friedman *Nature Med.* 2:144-147, 1996; Crystal *Science* 270:404-410, 1995;
Mulligan *Science* 260:926-932, 1993; Luo *et al. Nat. Biotechnol.* 18:33-37,2000; Behr
Acc. Chem. Res. 26:274-278, 1993; each of which is incorporated herein by reference).
However, for small-molecule-, protein-, and DNA-based therapeutics that require
25   intracellular administration and trafficking to the cytoplasm, there is an increasing
demand for new materials that facilitate *triggered* release in response to environmental
stimuli such as pH (Zauner *et al. Adv. Drug Del. Rev.* 30:97-113, 1998; incorporated
herein by reference). Following endocytosis, the pH within cellular endosomal
compartments is lowered, and foreign material is degraded upon fusion with lysosomal
30   vesicles (Kabanov *et al. Bioconjugate Chem.* 6:7-20, 1995; incorporated herein by
reference). New materials that release molecular payloads upon changes in pH within
the intracellular range and facilitate escape from hostile intracellular environments

could have a fundamental and broad-reaching impact on the administration of
hydrolytically- and/or enzymatically-labile drugs (Zauner *et al. Adv. Drug Del. Rev.*
30:97-113, 1998; Kabanov *et al. Bioconjugate Chem.* 6:7-20, 1995; each of which is
incorporated herein by reference). Herein, the fabrication of pH-responsive polymer

5   microspheres that release encapsulated contents quantitatively and essentially
instantaneously upon changes in pH within the intracellular range is reported.

The synthesis of poly(β-amino ester) **1** has been described above in Example 1
(Miller *Angew. Chem. Int. Ed.* 37:1768-1785, 1998; Hope *et al. Molecular Membrane
Technology* 15:1-14, 1998; Deshmukh *et al. New J. Chem.* 21:113-124, 1997; each of

10   which is incorporated herein by reference). Poly-1 is hydrolytically degradable, was
non-cytotoxic in initial screening assays, and is currently under investigation as a
synthetic vector for DNA delivery in gene therapy applications. The solubility of the
polymer in aqueous media is directly influenced by solution pH. Specifically, the solid,
unprotonated polymer is insoluble in aqueous media in the pH range 7.0 to 7.4, and the

15   transition between solubility and insolubility occurs at a pH around 6.5. Based on the
differences between extracellular and endosomal pH (7.4 and 5.0 - 6.5, respectively),
we hypothesized that microspheres formed from poly-1 might be useful for the
encapsulation and triggered release of compounds within the range of intracellular pH.

The encapsulation of therapeutic compounds within polymer microspheres is

20   often achieved employing a double emulsion process (O'Donnell *et al. Adv. Drug
Delivery Rev.* 28:25-42, 1997; incorporated herein by reference). The double emulsion
process is well established for the fabrication of microspheres from hydrophobic
polymers such as poly(lactic-*co*-glycolic acid) (PLGA), a biodegradable polymer
conventionally employed in the development of drug delivery devices (Anderson *et al.*

25   *Adv. Drug Delivery Rev.* 28:5-24, 1997; Okada *Adv. Drug Delivery Rev.* 28:43-70,
1997; each of which is incorporated herein by reference). Preliminary experiments
demonstrated the feasibility of the double emulsion process for the encapsulation of
water-soluble compounds using poly-1. Rhodamine-conjugated dextran was chosen as
a model for subsequent encapsulation and release studies for several reasons: 1)

30   rhodamine is fluorescent, allowing loading and release profiles to be determined by
fluorescence spectroscopy, 2) loaded microspheres could be imaged directly by
fluorescence microscopy, and 3) the fluorescence intensity of rhodamine is relatively

unaffected by pH within the physiological range (Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed., Molecular Probes, Inc., 1996, p. 29; incorporated herein by reference).

5　　　　　Microspheres encapsulating labeled dextran were fabricated from poly-1 and compared to controls formed from PLGA. The size distributions of microspheres formed from poly-1 correlated well with the distributions of PLGA microspheres within the range of 5-30 μm. Average particle sizes could be controlled by variations in experimental parameters such as homogenization rates and aqueous/organic solvent ratios (O'Donnell *et al. Adv. Drug Delivery Rev.* 28:25-42, 1997; incorporated herein

10　by reference). In contrast to PLGA microspheres, however, spheres formed from poly-1 aggregated extensively during centrifugation and washing steps (see Experimental Section above). Microspheres resuspended at pH 7.4 consisted primarily of large aggregates, and scanning electron microscopy (SEM) images revealed clusters of spheres that appeared to be physically joined or "welded" (data not shown).

15　　　　　It was found that aggregation could be eliminated if centrifugation and washing were conducted at reduced temperatures (4 °C), presumably due to the hardening of the polymer spheres at this lower temperature. SEM images of dextran-loaded poly-1 microspheres prepared in the 8-10 μm range revealed significant fracturing and the formation of large holes on their surfaces. Microspheres targeted in the range of 4-6

20　μm, however, were essentially free of cracks, holes, and other defects (Figure 6). Microspheres formulated for subsequent release experiments were fabricated in the smaller (< 6 μm) range. Encapsulation efficiencies for loaded poly-1 microspheres, determined by dissolving the spheres at pH 5.1 and measuring fluorescence intensity, were as high as 53%.

25　　　　　Suspensions of dried poly-1 microspheres at pH = 7.4 consisted primarily of single, isolated microspheres as determined by optical and fluorescence microscopy (Figure 8a). The zeta potential (ζ) of microparticle suspensions of poly-1 microspheres at pH 7 was +3.75 (± 0.62) mV, suggesting that the surfaces of the microspheres carry an overall positive charge at physiological pH. This could be relevant to the targeting

30　of these microspheres for cellular uptake, because net positive charges on particle surfaces may play a role in triggering endocytosis (Zauner *et al. Adv. Drug Delivery Rev.* 30:97-113, 1998; incorporated herein by reference).

Poly-1 microspheres suspended at pH 7.4 remained stable toward aggregation and degradation for several weeks (by visual inspection), but the microspheres dissolved instantly when the pH of the suspending medium was lowered between 5.1 and 6.5.

pH 7.4

pH < 6.5
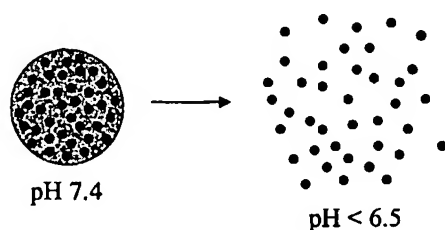
5

The release of labeled dextran from poly-1 microspheres was determined quantitatively by fluorescence microscopy (Figure 7). The release profile at pH 7.4 was characterized by a small initial burst in fluorescence (7-8%) which reached a limiting value of about 15% after 48 hours. This experiment demonstrated that the degradation of poly-1 was

10   relatively slow under these conditions and that greater than 90% of encapsulated material could be retained in the polymer matrix for suitably long periods of time at physiological pH.

To examine the application of poly-1 microspheres to the triggered release of encapsulated drugs in the endosomal pH range, we conducted a similar experiment in

15   which the pH of the suspension medium was changed from 7.4 to 5.1 during the course of the experiment. As shown in Figure 7, the microspheres dissolved rapidly when the suspension buffer was exchanged with acetate buffer (0.1 M, pH = 5.1), resulting in essentially *instantaneous* and *quantitative* release of the labeled dextran remaining in the polymer matrices. In sharp contrast, the release from dextran-loaded PLGA

20   microspheres *did not increase* for up to 24 hours after the pH of the suspending medium was lowered (Figure 7). Figure 8 shows fluorescence microscopy images of: (a) a sample of dextran-loaded microspheres at pH 7.4; and (b) a sample to which a drop of acetate buffer was added at the upper right edge of the microscope coverslip. The rapid release of rhodamine-conjugated dextran was visualized as streaking

25   extending from the dissolving microspheres in the direction of the diffusion of added acid and an overall increase in background fluorescence (elapsed time ≈ 5 seconds).

When targeting therapeutic compounds for intracellular delivery *via* endocytosis

or phagocytosis, it is not only important to consider a means by which the drug can be released from its carrier, but also a means by which the drug can escape endosomal compartments prior to being routed to lysosomal vesicles (Luo *et al. Nat. Biotechnol.* 18:33-37, 2000; Zauner *et al. Adv. Drug Delivery Rev.* 30:97-113, 1998; each of which

5   is incorporated herein by reference). One strategy for facilitating endosomal escape is the incorporation of weak bases, or "proton sponges," which are believed to buffer the acidic environment within an endosome and disrupt endosomal membranes by increasing the internal osmotic pressure within the vesicle (Demeneix *et al.*, in *Artificial Self-Assembling Systems for Gene Delivery* (Felgner *et al.*, Eds.), American

10   Chemical Society, Washington, D.C., 1996, pp. 146-151; incorporated herein by reference). Poly-1 microspheres are capable of releasing encapsulated material in the endosomal pH range *via* a mechanism (dissolution) that involves the protonation of amines in the polymer matrix. Thus, in addition to the rapid release of drug, poly-1 microspheres may also provide a membrane-disrupting means of endosomal escape,

15   enhancing efficacy by prolonging the lifetimes of hydrolytically unstable drugs contained in the polymer matrix.

Microspheres fabricated from poly-1 could represent an important addition to the arsenal of pH-responsive materials applied for intracellular drug delivery, such as pH-responsive polymer/liposome formulations (Gerasimov *et al. Adv. Drug Delivery*

20   *Rev.* 38:317-338, 1999; Linhart *et al. Langmuir* 16:122-127, 2000; Linhardt *et al. Macromolecules* 32:4457-4459, 1999; each of which is incorporated herein by reference). In contrast to many liposomal formulations, polymer microspheres are physically robust and can be stored dried for extended periods without deformation, decomposition, or degradation (Okada *Adv. Drug Delivery Rev.* 28:43-70, 1997;

25   incorporated herein by reference)—an important consideration for the formulation and packaging of new therapeutic delivery systems. The microspheres investigated in this current study fall within the size range of particles commonly used to target delivery to macrophages (Hanes *et al. Adv. Drug Delivery Rev.* 28:97-119, 1997; incorporated herein by reference). The rapid pH-release profiles for the poly-1 microspheres

30   described above may therefore be useful in the design of new DNA-based vaccines which currently employ PLGA as an encapsulating material (Singh *et al. Proc. Natl. Acad. Sci. USA* 97:811-816, 2000; Ando *et al. J. Pharm. Sci.* 88:126-130, 1999; Hedley

et al. Nat. Med. 4:365-368, 1998; each of which is incorporated herein by reference).

Example 3 - Accelerated Discovery of Synthetic Transfection Vectors: Parallel Synthesis and Screening of a Degradable Polymer Library

5

Introduction

The safe and efficient delivery of therapeutic DNA to cells represents a fundamental obstacle to the clinical success of gene therapy (Luo *et al. Nat. Biotechnol.* 18:33-37, 2000; Anderson *Nature* 392 Suppl.:25-30, 1996; each of which is

10   incorporated herein by reference). The challenges facing synthetic delivery vectors are particularly clear, as both cationic polymers and liposomes are less effective at mediating gene transfer than viral vectors. The incorporation of new design criteria has led to recent advances toward functional delivery systems (Lim *et al. J. Am. Chem. Soc.* 123:2460-2461, 2001; Lim *et al. J. Am. Chem. Soc.* 122:6524-6525, 2000; Hwang *et al.*

15   *Bioconjugate Chem.* 12:280-290, 2001; Putnam *et al. Proc. Natl. Acad. Sci. USA* 98:1200-1205, 2001; Benns *et al. Bioconjugate Chem.* 11:637-645, 2000; Midoux *et al. Bioconjugate Chem.* 10:406-411, 1999; each of which is incorporated herein by reference).   However, the paradigm for the development of polymeric gene delivery vectors remains the incorporation of these design elements into materials as part of an

20   iterative, linear process—an effective, albeit slow, approach to the discovery of new vectors. Herein, we report a parallel approach suitable for the synthesis of large libraries of degradable cationic polymers and oligomers and the discovery of new synthetic vector families through rapid cell-based screening assays (for a report on the parallel synthesis and screening of degradable polymers for tissue engineering, see:

25   Brocchini *et al. J. Am. Chem. Soc.* 119:4553-4554, 1997; incorporated herein by reference).

Experimental Section

General Considerations. All manipulations involving live cells or sterile materials

30   were performed in a laminar flow hood using standard sterile technique. Gel permeation chromatography (GPC) was performed using a Hewlett Packard 1100 Series isocratic pump, a Rheodyne Model 7125 injector with a 100-µL injection loop,

and two PL-Gel mixed-D columns in series (5 μm, 300 x 7.5 mm, Polymer Laboratories, Amherst, MA). THF/0.1M piperidine was used as the eluent at a flow rate of 1.0 mL/min. Data was collected using an Optilab DSP interferometric refractometer (Wyatt Technology, Santa Barbara, CA) and processed using the TriSEC

5   GPC software package (Viscotek Corporation, Houston, TX). The molecular weights and polydispersities of the polymers are reported relative to monodisperse polystyrene standards.

Materials. Primary amine and secondary amine monomers **1-20** were purchased from

10   Aldrich Chemical Company (Milwaukee, WI), Lancaster (Lancashire, UK), Alfa Aesar Organics (Ward Hill, MA), and Fluka (Milwaukee, WI). Diacrylate monomers **A-G** were purchased from Polysciences, Inc. (Warrington, PA), Alfa Aesar, and Scientific Polymer Products, Inc. (Ontario, NY). All monomers were purchased in the highest purity available (from 97% to 99+%) and were used as received without additional

15   purification. Plasmid DNA containing the firefly luciferase reporter gene (pCMV-Luc) was purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA) and used without further purification. (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Company (St. Louis, MO). Monkey kidney fibroblasts (COS-7 cells) used in transfection assays were purchased

20   from American Type Culture Collection (Manassas, VA) and grown at 37° C, 5% CO_2 in Dulbecco's modified Eagle's medium, 90%; fetal bovine serum, 10%; penicillin, 100 units/mL; streptomycin, 100 μg/mL. Luciferase detection kits used in high-throughput transfection assays were purchased from Tropix (Bedford, MA). All other materials and solvents were used as received without additional purification.

25

Synthesis of Polymer Library. All 140 polymerization reactions were conducted simultaneously as an array of individually labeled vials according to the following general protocol. Individual exceptions are noted where appropriate. Amine monomers **1-20** (2.52 mmol) were charged into appropriately labeled vials (as shown

30   below): liquid monomers were measured and transferred quantitatively using microliter pipettes; solid monomers were weighed directly into each vial. Anhydrous CH_2Cl_2 (1 mL) was added to each vial. One equivalent of liquid diacrylates **A-F** (2.52 mmol) was

added to each appropriate reaction vial using a microliter pipette, and the vial was capped tightly with a Teflon-lined cap. One equivalent of semi-solid diacrylate **G** was added to the appropriate vials as a solution in CH_2Cl_2 (2.52 mmol, 1 mL of a 2.52M solution in CH_2Cl_2) and the vials were tightly capped. An additional aliquot of CH_2Cl_2

5   (2 mL) was added to the reaction vials containing **19** and **20** to aid in the solubility of these monomers. The tightly capped vials were arrayed in two plastic test tube racks and secured to an orbital shaker in a 45 °C oven. (CAUTION: The heating of capped vials represents a possible explosion hazard. Oven temperature was monitored periodically for one week prior to the experiment to ensure reliable thermal stability.

10   Temperatures were found to vary within +/- 1 °C during this time period. Several test vials were monitored prior to conducting the larger experiment). The reaction vials were shaken vigorously at 45 °C for 5 days and allowed to cool to room temperature. Vials were placed in a large dessicator and placed under aspirator vacuum for 1 day and high vacuum for an additional 5 days to ensure complete removal of solvent. The

15   samples obtained were analyzed by GPC (55% of total library, see Table 2) and used directly in all subsequent screening experiments.

Table 2: GPC survey of 55% of the screening library showing molecular weights (M_w) and polydispersities (shown in parentheses).

	A	B	C	D	E	F	G
1	5900 (1.93)	4725 (1.89)			5220 (1.95)	1690 (1.74)	
2	6920 (1.87)	6050 (1.78)			5640 (1.85)		
3	6690 (1.79)	6050 (1.78)				2060 (1.76)	
4	7810 (2.49)	5720 (2.20)	9720 (2.49)	7960 (4.08)	7940 (3.25)		
5	10 800 (2.75)	5000 (2.50)	15 300 (3.17)	17 200 (6.91)	15 300 (3.92)	Insol.	9170 (2.50)
6	21 000 (3.70)	10 200 (3.4)		18 000 (6.06)			
7	14 300 (3.25)	11 880 (3.3)	20 200 (3.44)	10 300 (4.26)	15 500 (4.89)		22 500 (3.92)
8	2310 (1.62)	11 520 (3.60)		2230 (1.73)			
9	1010 (1.33)	2505 (1.67)	1240 (1.16)			Insol.	
10		<1000	Insol.				
11	6800 (1.91)	Insol.	9440 (1.79)	5550 (2.23)	6830 (1.93)	1990 (1.43)	6420 (1.75)
12	9310	9100	11 900	5810			12 300

	(2.06)	(2.53)	(2.18)	(1.77)			(1.85)
13	2990 (1.64)	3180 (2.12)	3680 (1.64)	2550 (1.82)	3230 (1.82)		3580 (1.64)
14	1350 (1.35)	3180 (2.12)	2110 (1.69)	1400 (1.4)	1752 (1.46)		2025 (1.62)
15		1550 (1.51)					
16		16 380 (2.60)					
17		8520 (2.13)		7290 (1.94)			
18		<1000					
19	12 400 (2.28)	18 445 (2.17)	39 700 (1.90)	17 400 (1.93)	14 800 (1.98)		13 900 (1.86)
20	16 900 (2.40)	46 060 (3.29)	49 600 (2.25)	30 700 (2.72)	18 700 (2.72)		17 100 (2.22)

Determination of Water Solubility. The solubilities of all samples sample were determined simultaneously at a concentration of 2 mg/mL in the following general manner. Each polymer sample (5 mg) was weighed into a 12 mL scintillation vial and 2.5 mL of acetate buffer (25 mM, pH = 5.0) was added to each sample using an a pipettor. Samples were shaken vigorously at room temperature for 1 hour. Each sample was observed visually to determine solubility.

Agarose Gel Electrophoresis Assay. The agarose gel electrophoresis assay used to determine the ability of polymers to form complexes with DNA was performed in the following manner. Using the solutions prepared in the above solubility assay (2 mg/mL in acetate buffer, 25 mM, pH = 5.0), stock solutions of the 70 water-soluble polymers were arrayed into a 96-well cell culture plate. DNA/polymer complexes were formed at a ratio of 1:5 (w/w) by transferring 10 µL of each polymer solution from the stock plate to a new plate using a multichannel pipettor. Each polymer was further diluted with 90 µL of acetate buffer (25 mM, pH = 5.0, total volume = 100 µL) and the plate was shaken for 30 seconds on a mechanical shaker. An aqueous solution of plasmid DNA (100 µL of a 0.04 µg/µL solution) was added to each well in the plate and the solutions were vigorously mixed using a multichannel pipettor and a mechanical shaker. DNA/polymer complexes were formed at a ratio of 1:20 (w/w) in the same manner with the following exceptions: 40 µL of polymer stock solution was transferred to a new plate and diluted with 60 µL of acetate buffer (total volume = 100 µL) prior to

adding the aqueous DNA solution (100 μL). DNA/polymer complexes were incubated at room temperature for 30 minutes, after which samples of each solution (15 μL) were loaded into a 1% agarose gel (HEPES, 20 mM, pH = 7.2, 500 ng/mL ethidium bromide) using a multichannel pipettor. NOTE: Samples were loaded on the gel with a

5   loading buffer consisting of 10% Ficoll 400 (Amersham Pharmacia Biotech, Uppsala, Sweden) in HEPES (25 mM, pH = 7.2). Bromphenol blue was not included as a visual indicator in the loading buffer, since this charged dye appeared to interfere with the complexation of polymer and DNA. Samples were loaded according to the pattern shown in Figure 9, such that samples corresponding to DNA/polymer ratios of 1:5 and

10   1:20 were assayed in adjacent positions on the gel. The gel was run at 90V for 30 minutes and DNA bands were visualized by ethidium bromide staining.

General Protocol for Cell Transfection Assays. Transfection assays were performed in triplicate in the following general manner. COS-7 cells were grown in 96-well plates

15   at an initial seeding density of 15,000 cells/well in 200 μL of phenol red-free growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin 100 units/mL, streptomycin 100 μg/mL). Cells were grown for 24 hours in an incubator, after which the growth medium was removed and replaced with 200 μL of Optimem medium (Invitrogen Corp., Carlsbad, CA) supplemented with HEPES (total

20   concentration = 25 mM). Polymer/DNA complexes prepared from the 56 water-soluble/DNA-complexing polymers previously identified were prepared as described above at a ratio of 1:20 (w/w)) using a commercially available plasmid containing the firefly luciferase reporter gene (pCMV-Luc). An appropriate volume of each sample was added to the cells using a multichannel pipettor (e.g., 15 μL yielded 300 ng

25   DNA/well; 30 μL yielded 600 ng DNA/well). Controls employing poly(ethylene imine) (PEI) and polylysine, prepared at DNA/polymer ratios of 1:1, were prepared in a similar manner and included with DNA and no-DNA controls. Controls employing Lipofectamine 2000 (Invitrogen Corp.) were performed at several concentrations (0.1, 0.2, 0.4, and 0.6 μL) as described in the technical manual for this product

30   (http://lifetechnologies.com). Cells were incubated for 4 hours, after which the serum-free growth medium was removed and replaced with 100 μL of phenol-red-free growth medium. Cells were incubated for an additional period of time (typically varied

between 36 to 60 hours) and luciferase expression was determined using a
commercially available assay kit (Tropix, Inc., Bedford, MA). Luminescence was
quantified in white, solid-bottom polypropylene 96-well plates using a 96-well
bioluminescence plate reader. Luminescence was expressed in relative light units and
5   was not normalized to total cell protein in this assay.

Results and Discussion

Poly(β-amino ester)s are hydrolytically degradable, condense plasmid DNA at
physiological pH, and are readily synthesized via the conjugate addition of primary or
10   secondary amines to diacrylates (Eq. 1 and 2) (Lynn *et al. J. Am. Chem. Soc.*
122:10761-10768, 2000; incorporated herein by reference).   An initial screen of
model polymers identified these materials as potential gene carriers and demonstrated
that structural variations could have a significant impact on DNA binding and
transfection efficacies (Lynn *et al. J. Am. Chem. Soc.* 122:10761-10768, 2000;
15   incorporated herein by reference). We reasoned that this approach provided an
attractive framework for the elaboration of large libraries of structurally-unique
polymers for several reasons: 1) diamine and diacrylate monomers are inexpensive,
commercially available starting materials, 2) polymerization can be accomplished
directly in a single synthetic step, and 3) purification steps are generally unnecessary as
20   no byproducts are generated during polymerization.
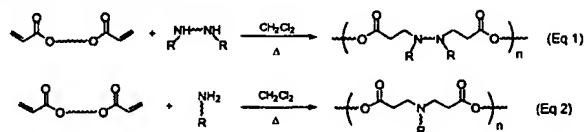
The paucity of commercially available bis(secondary amines) limits the degree
of structural diversity that can be achieved using the above synthetic approach.
However, the pool of useful, commercially available monomers is significantly
25   expanded when primary amines are considered as potential library building blocks.
Because the conjugate addition of amines to acrylate groups is generally tolerant of
functionalities such as alcohols, ethers, and tertiary amines (Ferruti *et al. Adv. Polym.
Sci.* 58:55-92, 1984; incorporated herein by reference), we believed that the
incorporation of functionalized primary amine monomers into our synthetic strategy
30   would serve to broaden structural diversity. Diacrylate monomers **A-G** and amine

monomers **1-20** were selected for the synthesis of an initial screening library.
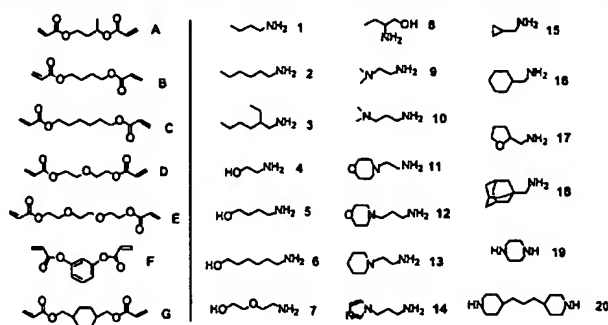
The size of the library constructed from this set of monomers (7 diacrylates x 20 amines = 140 structurally-unique polymers) was chosen to be large enough to incorporate sufficient diversity, yet small enough to be practical without the need for automation in our initial studies. It was unclear at the outset whether a polymer such as **G16** (formed from hydrophobic and sterically bulky monomers **G** and **16**) would be water-soluble at physiological pH or be able to condense DNA sufficiently. However, monomers of this type were deliberately incorporated to fully explore diversity space, and in anticipation that this library may ultimately be useful as a screening population for the discovery of materials for applications other than gene delivery (For a report on the parallel synthesis and screening of degradable polymers for tissue engineering, see: Brocchini *et al. J. Am. Chem. Soc.* 119:4553-4554, 1997, incorporated herein by reference; Lynn *et al. Angew. Chem. Int. Ed.* 40:1707-1710, 2001; incorporated herein by reference).

Polymerization reactions were conducted simultaneously as an array of individually labeled vials. Reactions were performed in methylene chloride at 45 °C for 5 days, and polymers were isolated by removal of solvent to yield 600-800 mg of each material. Reactions performed on this scale provided amounts of each material sufficient for routine analysis by GPC and all subsequent DNA-binding, toxicity, and transfection assays. A survey of 55% of the library by GPC indicated molecular weights ranging from 2000 to 50 000 (relative to polystyrene standards). As high molecular weights are not required for DNA-complexation and transfection (as shown below) (Kabanov *et al.*, in *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial*, John Wiley and Sons, New York, 1998; incorporated herein by reference), this library provided a collection of polymers and oligomers

suitable for subsequent screening assays.

Of the 140 members of the screening library, 70 samples were sufficiently water-soluble (2 mg/mL, 25 mM acetate buffer, pH = 5.0) to be included in an electrophoretic DNA-binding assay (Figure 9). To perform this assay as rapidly and

5   efficiently as possible, samples were mixed with plasmid DNA at ratios of 1:5 and 1:20 (DNA/polymer, w/w) in 96-well plates and loaded into an agarose gel slab capable of assaying up to 500 samples using a multi-channel pipettor. All 70 water-soluble polymer samples were assayed simultaneously at two different DNA/polymer ratios in less than 30 minutes. As shown in Figure 9, 56 of the 70 water-soluble polymer

10   samples interacted sufficiently with DNA to retard migration through the gel matrix (e.g., **A4** or **A5**), employing the 1:20 DNA/polymer ratio as an exclusionary criterion. Fourteen polymers were discarded from further consideration (e.g., **A7** and **A8**), as these polymers did not complex DNA sufficiently.

The DNA-complexing materials identified in the above assay were further

15   investigated in transfection assays employing plasmid DNA and the COS-7 cell line. All assays were performed simultaneously with the firefly luciferase reporter gene (pCMV-Luc) and levels of expressed protein were determined using a commercially available assay kit and a 96-well luminescence plate reader. Figure 10 displays data generated from an assay employing pCMV-Luc (600 ng/well) at DNA/poly ratios of

20   1:20 (w/w). The majority of the polymers screened did not mediate transfection above a level typical of "naked" DNA (no polymer) controls under these conditions. However, several wells expressed higher levels of protein and the corresponding polymers were identified as "hits" in this assay. Polymers **B14** ($M_w = 3180$) and **G5** ($M_w = 9170$), for example, yielded transfection levels 4-8 times higher than control experiments

25   employing poly(ethylene imine) (PEI), a polymer conventionally employed as a synthetic transfection vector (Boussif et al. Proc. Natl. Acad. Sci. USA 92:7297-7301, 1995; incorporated herein by reference), and transfection levels within or exceeding the range of expressed protein using Lipofectamine 2000 (available from Invitrogen Corp. (Carlsbad, CA)), a leading commercially available lipid-based transfection vector

30   system. Polymers **A14**, **C5**, **G7**, **G10**, and **G12** were also identified as positive "hits" in the above experiment, but levels of gene expression were lower than **B14** and **G5**.

Structural differences among synthetic polymers typically preclude a general set

of optimal transfection conditions. For example, polymers **C5**, **C14**, and **G14** were toxic at the higher concentrations employed above (Determined by the absence of cells in wells containing these polymers as observed upon visual inspection. These polymers were less toxic and mediated transfection at lower concentration.), but mediated

5 transfection at lower DNA and polymer concentrations (data not shown). The assay system described above can easily be modified to evaluate polymers as a function of DNA concentration, DNA/polymer ratio, cell seeding densities, or incubation times. Additional investigation will be required to more fully evaluate the potential of this screening library, and experiments to this end are currently underway.

10 The assays above were performed in the absence of chloroquine, a weak base commonly added to enhance *in vitro* transfection (Putnam *et al. Proc. Natl. Acad. Sci. USA* 98:1200-1205, 2001; Benns *et al. Bioconjugate Chem.* 11:637-645, 2000; Midoux *et al. Bioconjugate Chem.* 10:406-411, 1999; Kabanov *et al.*, in *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial*, John Wiley and

15 Sons, New York, 1998; each of which is incorporated herein by reference), and the results therefore reflect the intrinsic abilities of those materials to mediate transfection. The polymers containing monomer **14** are structurally similar to other histidine containing "proton sponge" polymers (Putnam *et al. Proc. Natl. Acad. Sci. USA* 98:1200-1205, 2001; Benns *et al. Bioconjugate Chem.* 11:637-645, 2000; Midoux *et al.*

20 *Bioconjugate Chem.* 10:406-411, 1999; each of which is incorporated herein by reference), and could enhance transfection by buffering acidic intracellular compartments and mediating endosomal escape (Putnam *et al. Proc. Natl. Acad. Sci. USA* 98:1200-1205, 2001; Benns *et al. Bioconjugate Chem.* 11:637-645, 2000; Midoux *et al. Bioconjugate Chem.* 10:406-411, 1999; Boussif *et al. Proc. Natl. Acad. Sci. USA*

25 92:7297-7301, 1995; each of which is incorporated herein by reference). The efficacy of polymers containing monomer **5** is surprising in this context, as these materials do not incorporate an obvious means of facilitating endosomal escape. While the efficacy of these latter polymers is not yet understood, their discovery helps validate our parallel approach and highlights the value of incorporating structural diversity, as these

30 polymers may not have been discovered on an *ad hoc* basis. Polymers incorporating hydrophilic diacrylates **D** and **E** have not produced "hits" under any conditions thus far, providing a possible basis for the development of more focused libraries useful for the

elucidation of structure/activity relationships.

We have generated a library of 140 degradable polymers and oligomers useful for the discovery of new DNA-complexing materials and gene delivery vectors. Several of these materials are capable of condensing DNA into structures small enough to be

5   internalized by cells and release the DNA in a transcriptionally active form. The total time currently required for library design, synthesis, and initial screening assays is approximately two weeks. However, the incorporation of robotics and additional monomers could significantly accelerate the pace at which new DNA-complexing materials and competent transfection vectors are identified.

10

Example 4 – Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery

15   One of the major barriers to the success of gene therapy in the clinic is the lack of safe and efficient methods of delivering nucleic acids. Currently, the majority of clinical trials use modified viruses as delivery vehicles, which, while effective at transferring DNA to cells, suffer from potentially serious toxicity and production problems (Somia *et al. Nat Rev Genet.* 1:91 (2000); incorporated herein by reference).

20   In contrast, non-viral systems offer a number of potential advantages, including ease of production, stability, low immunogenicity and toxicity, and reduced vector size limitations (Ledley *Human Gene Therapy* 6:1129 (1995); incorporated herein by reference). Despite these advantages, however, existing non-viral delivery systems are far less efficient than viral vectors (Luo *et al. Nature Biotechnology* 18:33 (2000);

25   incorporated herein by reference).

One promising group of non-viral delivery compounds are cationic polymers, which spontaneously bind and condense DNA. A wide variety of cationic polymers that transfect *in vitro* have been characterized, both natural, such as protein (Fominaya *et al. Journal of Biological Chemistry* 271:10560 (1996); incorporated herein by

30   reference) and peptide systems (Schwartz *et al. Curr Opin Mol Ther.* 2:162 (2000); incorporated herein by reference), and synthetic polymers such as poly(ethylene imine) (Boussif *et al. Proceedings of the National Academy of Sciences of the United States of*

America 92:7297 (1995);incorporated herein by reference) and dendrimers (Kabanov *et al. Self-assembling complexes for gene delivery: from laboratory to clinical trial*, Wiley, Chichester ; New York, 1998; incorporated herein by reference). Recent advances in polymeric gene delivery have in part focused on making the polymers

5   more biodegradable to decrease toxicity. Typically, these polymers contain both chargeable amino groups, to allow for ionic interactions with the negatively charged phosphate backbone of nucleic acids, and a biodegradable linkage such as a hydrolyzable ester linkage. Several examples of these include poly(alpha-(4-aminobutyl)-L-glycolic acid) (Lim *et al. Journal of the American Chemical Society*

10   122:6524 (2000); incorporated herein by reference), network poly(amino ester) (Lim *et al. Bioconjugate Chemistry* 13:952 (2002); incorporated herein by reference), and poly(beta-amino ester)s (Lynn *et al. Journal of the American Chemical Society* 122:10761 (2000); Lynn *et al. Journal of the American Chemical Society* 123:8155 (2001); each of which is incorporated herein by reference). Poly(beta-amino ester)s are

15   particularly interesting because they show low cytotoxicity and are easily synthesized via the conjugate addtion of a primary amine or bis(secondary amine) to a diacrylate (Figure 11) (Lynn *et al. Journal of the American Chemical Society* 122:10761 (2000); Lynn *et al. Journal of the American Chemical Society* 123:8155 (2001); each of which is incorporated herein by reference).

20   Traditional development of new biomedical polymers has been an iterative process. Polymers were typically designed one at a time and then individually tested for their properties. More recently, attention has focused on the development of parallel, combinatorial approaches that facilitate the generation of structurally-diverse libraries of polymeric biomaterials (Brocchini *Advanced Drug Delivery Reviews* 53:123

25   (2001); incorporated herein by reference). This combinatorial approach has also been applied to the discovery of gene delivery polymers. For example, Murphy *et al.* generated a targeted combinatorial library of 67 peptoids via solid-phase synthesis and screened them to identify new gene delivery agents (Murphy *et al. Proceedings of the National Academy of Sciences of the United States of America* 95:1517 (1998);

30   incorporated herein by reference).

     In this Example is described new tools for high-throughput, parallel combinatorial synthesis and cell-based screening of a large library of 2350 structurally

diverse, poly(beta-amino ester)s. This approach allows for the screening of polymers in cell-based assays without the polymers ever leaving the solution phase following synthesis. This approach combined with the use of robotic fluid handling systems allows for the generation and testing of thousands of synthetic polymers in cell-based

5   assays in a relatively short amount of time. Using this approach, 46 new polymers that perform as well or better than conventional non-viral delivery systems such as poly(ethylene imine) have been identified.

Results and Discussion

10   *High-throughput polymer synthesis.* The primary factors limiting the throughput and automation of poly(beta-amino esters) synthesis and testing was the viscosity of monomer and polymer solutions, and the difficulty with manipulating the solid polymer products. While automation of liquid handling is straightforward using conventional robotics, the manipulation of solids and viscous liquids on a small scale is

15   not. Therefore, a system was developed in which polymers could be synthesized and screened in cell-based assays without leaving the solution phase. Since this would require the presence of residual solvent in the cell assays, the relatively non-toxic solvent, dimethyl sulfoxide (DMSO) was chosen. DMSO is a commonly used solvent in cell culture and is routinely used in storing frozen stocks of cells. DMSO is miscible

20   with water and is generally well tolerated in living systems.

The first step in preparing for high-throughput synthesis was to identify conditions that would allow for the production of polymer yet possess a manageable viscosity. Small scale, pilot experiments showed that polymerization could be performed effectively at 1.6 M in DMSO at 56°C for 5 days. Based on these

25   experiments, a general strategy was developed for polymer synthesis and testing. All monomers (Figure 12) were diluted to 1.6 M in DMSO, and then using both a fluid-handling robot and a 12 channel micropipettor, we added 150 microliters of each amine and diacrylate monomer into a polypropylene deep well plate and then sealed it with aluminum foil. The plates were placed on an orbital shaker and incubated at 56°C for 5

30   days. To compensate for the increased viscosity of polymeric solutions, 1 ml of DMSO was added to each well of the plate, and the plates were then stored at 4°C until further use. These methods allos for 2350 reactions in a single day. Furthermore, the

production and storage of polymers in a 96-well format allowed for an easy transition
into 96-well format cell-based testing of polymer transfection efficiency.

High-throughput polymer testing. Once synthesized, all polymers were tested for their
ability to deliver the Lucieferase expressing plasmid, pCMV-luc, into the monkey

5   kidney fibroblast cell line COS-7. Due to the large size of the polymer library, a high-
throughput method for cell based screening of transfection efficience was developed.
Since polymers were stored in 96 well plates, all polymer dilutions, DNA
complexation, and cell transfections were performed in parallel by directly transferring
polymers from plate to plate using a liquid handling robot. All polymers were

10   synthesized using the same concentration of amine monomer, thus comparison between
polymers at a fixed amine monomer:DNA phosphate ratio (N:P ratio) was
straightforward. While the amine monomers contain either one, two, or three amines
per monomer, initial broad-based screens for transfection efficiency were greatly
simplified by maintaining a constant monomer concentration in all wells of a single

15   plate, and therefore a constant volume of polymer solution per reaction (see methods
below).

The efficiency of *in vitro* transfection using cationic polymers such as
poly(ethylene imine) is very sensitive to the ratio of polymer to DNA present during
complex formation (Gebhart *et al. Journal of Controlled Release* 73:401 (2001);

20   incorporated herein by reference). N:P rations of 10:1, 20:1, and 40:1 were selected for
our initial screens based on previous experience with these types of polymers. Using
our high-throughput system, we screened all 2350 polymers at these three ratios.
Transfection values at the best condition for each polymer were tabulated into a
histogram (Figure 13). These results were compared to three controls: naked DNA (no

25   transfection agent), poly(ethylene imine) (PEI), and Lipofectamine 2000. The low,
residual levels of DMSO present in the transfection solutions did not affect transfection
efficiency of either naked DNA or PEI. Thirty-three of the 2350 polymers were found
to be as good or better than PEI in this unoptimized system.

Since cationic polymer transfections tend to be sensitive to polymer:DNA ratio,

30   we decided to optimize transfection conditions with the best polymers from our
preliminary screen. Using the results above as a rough guide, the transfection condition
for the best 93 polymers were optimized by testing them at N:P ratios above and below

the optimal transfection conditions identified in the broad based screen. In order to develop a high-throughput optimization system, these were tested using an N:P ratio multiplier system to simplify simultaneous testing and plate-to-plate transfer. Starting with the best N:P ratio identified in the preliminary screen, the polymers were retested

5　　at six N:P ratios equal to the best ratio times 0.5, 0.75, 1.0, 1.25, 1.5, and 1.75, in triplicate. For example, if the optimal ratio identified in the screen for a given polymer was 20:1, then that polymer was rescreened in triplicate at N:P ratios of 10:1, 15:1, 20:1, 25:1, 30:1, and 35:1. The average transfection efficiencies with standard deviation from the best condition for the best 50 polymers are shown in Figure 14,

10　　along with control data. In this experiment, 46 polymers were identified that transfect as good or better than PEI. All 93 of these polymers were also tested for their ability to bind DNA using agarose gel electrophoresis (Figure 15). Interestingly, while almost all of the polymers bind DNA as expected, two polymers that transfect at high levels do not: M17 and KK89 (Figure 14).

15　　　　　To further examine the transfection properties of these polymers, ten high transfecting polymers were tested for their ability to deliver the green fluorescent protein plasmid, pCMV-eGFP. Unlike pCMV-luc, pCMV-eGFP provides information concerning what percentage of cells is transfected. High levels of transfection were observed for all 10 polymers, and two of the best are shown in Figure 16.

20　　　　　The "hits" identified in the above assays reveal a surprisingly diverse and unexpected collection of polymers. Particularly surpising is the large collection of hydrophobic, D-monomer-based polymers. In fact, the diacrylate monomers used to make the best performing 50 polymers are almost always hydrophobic. Further analysis reveals two more common features of the effective polymers: 1) twelve of the

25　　26 polymers that are better than the best conventional reagent, Lipofectamine 2000, have mono- an di-alcohol side groups, and 2) linear, bis(secondary amines) are also prevelant in the hit structures. Also surprising was the identification of two polymers that transfect at high levels but do not appear to bind DNA (KK89 and M17). Both are also insoluble at pH 5 and pH 7. Their ability to facilitate DNA uptake may be due to

30　　permeabilization of the cellular membrane.

　　　　　Also important for the function of gene delivery polymers is length (Remy *et al.*
Advanced Drug Delivery Reviews 30:85 (1998); Schaffer *et al. Biotechnol Bioeng*

67:598 (2000); each of which is incorporated herein by reference). Using these results as a framework, a range of polymer lengths for each hit polymer may be prepared by carefully varying relative monomer concentrations. Evidence shows that (1) like PEI, poly(beta-amino ester) length is important in the gene delivery proficiency of these

5   polymers, and (2) that the hits identified here can be resynthesized using conventional methods and still deliver DNA effectively.

Experimental Protocols

Polymer synthesis. Monomers were purchased from Aldrich (Milwaukee, WI), TCI

10   (Portland, OR), Pfaltz & Bauer (Waterbury, CT), Matrix Scientific (Columbia, SC), Acros-Fisher (Pittsburg, PA), Scientific Polymer (Ontario, NY), Polysciences (Warrington, PA), and Dajac monomer-polymer (Feasterville, PA). These were dissolved in DMSO (Aldrich) to a final concentration of 1.6 M. All possible pair wise combinations amine and diacrylate monomers were added in 150 µl aliquots to each

15   well of 2 ml 96 well polypropylene masterblock deep well plates (Griener America, Longwood, FL). The plates were sealed with aluminum foil, and incubated at 56°C while rotating on an orbital shaker. After 5 days, 1 ml of DMSO was added to each well, and the plates were resealed and stored frozen at 4°C until ready to be used. *Transfection experiments.* 14,000 cos-7 cells (ATCC, Manassas, VA) were seeded into

20   each well of a solid white or clear 96 well plate (Corning-Costar, Kennebunk, ME) and allowed to attached overnight in growth medium, composed of: 500 ml phenol red minus DMEM, 50 ml heat inactivated FBS, 5 ml penicillin/streptomycin (Invitrogen, Carlsbad, CA). Each well of a master block 96-well plate was filled with 1 ml of 25 mM sodium acetate pH 5. To this, 40 µl, 20 µl, or 10 µl of the polymer/DMSO solution

25   was added. 25 µl of the diluted polymer was added to 25 µl of 60 µg/ml pCMV-luc DNA (Promega, Madison, WI) or pEGFP-N1 (Invitrogen) in a half volume 96 well plate. These were incubated for 10 minutes, and then 30 µl of the polymer-DNA solution was added to 200 µl of Optimem with sodium bicarbonate (Invitrogen) in 96 well polystyrene plates. The medium was removed from the cells using a 12-channel

30   wand (V & P Scientific, San Diego, CA) after which 150 µl of the optimem-polymer-DNA solution was immediately added. Complexes were incubated with the cells for 1 hour and then removed using the 12-channel wand and replaced with 105 µl of growth

medium. Cells were allowed to grow for three days at 37°C, 5% CO_2 and then analyzed for luminescence (pCMV-luc) or fluorescence (pEGFP-N1). Control experiments were performed by as described above, but using poly(ethylene imine) MW 25,000 (Aldrich) replacing synthesized polymer, and at polymer:DNA weight

5   ratios of 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, and 2:1. Lipofectamine 2000 (Invitrogen) transfections were performed as described by the vendor, except that complexes were removed after 1 hour.

Luminescence was analyzed using bright-glo assay kits (Promega). Briefly, 100 μl of bright-glo solution was added to each well of the microtiter plate containing

10   media and cells. Luminescence was measured using a Mithras Luminometer (Berthold, Oak Ridge, TN). In some cases, a neutral density filter (Chroma, Brattleboro, VT) was used to prevent saturation of the luminometer. A standard curve for Luciferase was generated by titration of Luciferase enzyme (Promega) into growth media in white microtiter plates. eGFP expression was examined using a Zeiss Aciovert 200 inverted

15   microscope.

Agarose gel electrophoresis DNA-binding assays were done at a N:P ratio of 40:1, as previously described (Lynn *et al. Journal of the American Chemical Society* 123:8155 (2001); incorporated herein by reference). All liquid handling was performed using an EDR384S/96S robot (Labcyte, Union City, CA) or a 12 channel

20   micropippettor (Thermo Labsystems, Vantaa, Finland) in a laminar flow hood.

Example 5-Synthesis of Poly(beta-amino esters) Optimized for Highly Effective Gene Delivery

25

The effect of molecular weight, polymer/DNA ratio, and chain end-group on the transfection properties of two unique poly(β-amino ester) structures was determined. These factors can have a dramatic effect on gene delivery function. Using high throughput screening methods, poly(β-amino esters) that transfect better than PEI

30   and Lipofectamine 2000 (two of the best commercially available transfection reagents) have been discovered.

Materials and Methods

Polymer Synthesis. Poly-1 and Poly-2 polymers were synthesized by adding 1,4-butanediol diacrylate (99+%) and 1,6-hexanediol diacrylate (99%), respectively, to 1-amino butanol (98%). These monomers were purchased from Alfa Aesar (Ward Hill,

5   MA). Twelve versions each of Poly-1 and Poly-2 were generated by varying the amine/diacrylate stoichiometric ratio. To synthesize each of the 24 unique polymers, 400 mg of 1-amino butanol was weighed into an 8 mL sample vial with Telfon-lined screw cap. Next, the appropriate amount of diacrylate was added to the vial to yield a stoichiometric ratio between 1.4 and 0.6. A small Telfon-coated stir bar was then put in

10   each vial. The vials were capped tightly and placed on a multi-position magnetic stir-plate residing in an oven maintained at 100 °C. After a reaction time of 5 hr, the vials were removed from the oven and stored at 4 °C. All polymers were analyzed by GPC.

Gel Permeation Chromatography (GPC). GPC was performed using a Hewlett

15   Packard 1100 Series isocratic pump, a Rheodyne Model 7125 injector with a 100-μL injection loop, and a Phenogel MXL column (5μ mixed, 300 x 7.5 mm, Phenomenex, Torrance, CA). 70% THF/30% DMSO (v/v) + 0.1 M piperidine was used as the eluent at a flow rate of 1.0 mL/min. Data was collected using an Optilab DSP interferometric refractometer (Wyatt Technology, Santa Barbara, CA) and processed using the TriSEC

20   GPC software package (Viscotek Corporation, Houston, TX). The molecular weights and polydispersities of the polymers were determined relative to monodisperse polystyrene standards.

Luciferase Transfection Assays. COS-7 cells (ATCC, Manassas, VA) were seeded

25   (14,000 cells/well) into each well of an opaque white 96-well plate (Corning-Costar, Kennebunk, ME) and allowed to attach overnight in growth medium. Growth medium was composed of 90% phenol red-free DMEM, 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). To facilitate handling, polymers stock solutions (100 mg/mL) were prepared in DMSO solvent. A small

30   residual amount of DMSO in the transfection mixture does not affect transfection efficiency and does not result in any observable cytotoxicity. Working dilutions of each polymer were prepared (at concentrations necessary to yield the different

polymer/DNA weight ratios) in 25 mM sodium acetate buffer (pH 5). 25 μl of the
diluted polymer was added to 25 μl of 60μg/ml pCMV-Luc DNA (Elim
Biopharmaceuticals, South San Francisco, CA) in a well of a 96-well plate. The
mixtures were incubated for 10 minutes to allow for complex formation, and then 30 μl

5   of the each of the polymer-DNA solutions were added to 200 μl of Opti-MEM with
sodium bicarbonate (Invitrogen) in 96-well polystyrene plates. The growth medium
was removed from the cells using a 12-channel aspirating wand (V&P Scientific, San
Diego, CA) after which 150 μl of the Opti-MEM-polymer-DNA solution was
immediately added. Complexes were incubated with the cells for 1 hr and then

10   removed using the 12-channel wand and replaced with 105 μl of growth medium. Cells
were allowed to grow for three days at 37°C, 5% CO_2 and were then analyzed for
luciferase expression. Control experiments were also performed with PEI (MW =
25,000, Sigma-Aldrich) and Lipofectamine 2000 (Invitrogen). PEI transfections were
performed as described above, but using polymer:DNA weight ratios of 1:1.

15   Lipofectamine 2000 transfections were performed as described by the vendor, except
that complexes were removed after 1 hour.

    Luciferase expression was analyzed using Bright-Glo assay kits (Promega).
Briefly, 100 μl of Bright-Glo solution was added to each well of the 96-well plate
containing media and cells. Luminescence was measured using a Mithras

20   Luminometer (Berthold, Oak Ridge, TN). A 1% neutral density filter (Chroma,
Brattleboro, VT) was used to prevent saturation of the luminometer. A standard curve
for Luciferase was generated by titration of Luciferase enzyme (Promega) into growth
media in an opaque white 96-well plate.

    Measurement of Cytotoxicity. Cytotoxicity assays were performed in the same manner

25   as the luciferase transfection experiments with the following exception. Instead of
assaying for luciferase expression after 3 days, cells were assayed for metabolic activity
using the MTT Cell Proliferation Assay kit (ATCC) after 1 day. 10 μL of MTT
Reagent was added to each well. After 2 hr incubation at 37 °C, 100 μL of Detergent
Reagent was added to each well. The plate was then left in the dark at room

30   temperature for 4 hr. Optical absorbance was measured at 570 nm using a SpectaMax

190 microplate reader (Molecular Devices, Sunnyvale, CA) and converted to % viability relative to control (untreated) cells.

Cellular Uptake Experiments. Uptake experiments were done as previously described, with the exception that a 12-well plate format was used instead of a 6-well plate format

5   (Akinc, A., et al., *Parallel synthesis and biophysical characterization of a degradable polymer library of gene delivery.* J. Am. Chem. Soc., 2003; incorporated herein by reference). COS-7 cells were seeded at a concentration of 1.5×10^5 cells/well and grown for 24 hours prior to performing the uptake experiments. Preparation of polymer/DNA complexes was done in the same manner as in the luciferase transfection

10 experiments, the only differences being an increase in scale (2.5 μg DNA per well of 12-well plate as opposed to 600 ng DNA per well of 96-well plate) and the use of Cy5-labeled plasmid instead of pCMV-Luc (Akinc, A. and R. Langer, *Measuring the pH environment of DNA delivered using nonviral vectors: Implications for lysosomal trafficking.* Biotechnol. Bioeng., 2002. 78(5): p. 503-8; incorporated herein by

15 reference). As in the transfection experiments, complexes were incubated with cells for 1 hr to allow for cellular uptake by endocytosis. The relative level of cellular uptake was quantified using a flow cytometer to measure the fluorescence of cells loaded with Cy5-labeled plasmid.

20 *GFP Transfections.* GFP transfections were carried in COS-7 (green monkey kidney), NIH 3T3 (murine fibroblast), HepG2 (human hepatocarcinoma), and CHO (Chinese Hamster Ovary) cell lines. All cell lines were obtained from ATCC (Manassas, VA) and maintained in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere. Cells were seeded on 6-well

25 plates and grown to roughly 80-90% confluence prior to performing the transfection experiments. Polymer/DNA complexes were prepared as described above using the pEGFP-N1 plasmid (Clontech, Palo Alto, CA) (5 μg/well). Complexes were diluted in 1 mL Opti-MEM and added to the wells for 1 hr. The complexes were then removed and fresh growth media was added to the wells. After 2 days, cells were harvested and

30 analyzed for GFP expression by flow cytometry. Propidium iodide staining was used to exclude dead cells from the analysis.

Flow Cytometry. Flow cytometry was performed with a FACSCalibur (Becton Dickinson) equipped with an argon ion laser capable of exciting GFP (488 nm excitation) and a red diode laser capable of exciting Cy5 (635 nm excitation). The

5   emission of GFP was filtered using a 530 nm band pass filter and the emission of Cy5 was filtered using a 650 long pass filter. The cells were appropriately gated by forward and side scatter and 30,000 events per sample were collected.

Results and Discussion

10   *Polymer Synthesis.* As previously described (Lynn, D.M. and R. Langer, *Degradable poly(β-amino esters): synthesis, characterization, and self-assembly with plasmid DNA.* J. Am. Chem. Soc., 2000. 122(44): p. 10761-10768; incorporated herein by reference), the synthesis of poly(β-amino esters) proceeds *via* the conjugate addition of amines to acrylate groups. Because the reaction is a step polymerization, a broad,

15   statistical distribution of chain lengths is obtained, with average molecular weight and chain end-groups controlled by monomer stoichiometry (Flory, P., in *Principles of Polymer Chemistry.* 1953, Cornell University Press: Ithaca, NY. p. 40-46, 318-323; Odian, G., *Step Polymerizaton*, in *Principles of Polymerization.* 1991, John Wiley & Sons, Inc.: New York. p. 73-89; each of which is incorporated herein by reference).

20   Molecular weight increases as the ratio of monomers nears stoichiometric equivalence, and an excess of amine or diacrylate monomer results in amine- or acrylate-terminated chains, respectively. For this class of polymers, precise control of stoichiometry is essential for controlling polymer molecular weight. While monomer stoichiometry is the most important factor affecting chain length, consideration should also be given to

25   competing side reactions that can impact the molecular weight and structure of polymer products. In particular, intramolecular cyclization reactions, where an amine on one end of the growing polymer chain reacts with an acrylate on the other end, can limit obtained molecular weights (Odian, G., *Step Polymerizaton*, in *Principles of Polymerization.* 1991, John Wiley & Sons, Inc.: New York. p. 73-89; incorporated

30   herein by reference). These cyclic chains may also have properties that differ from those of their linear counterparts.

In this work, we have modified the previously reported polymerization

procedure in order to better control monomer stoichiometry and to minimize
cyclization reactions. First, the scale of synthesis was increased from roughly 0.5 g to 1
g to allow for control of stoichiometry within 1%. Further improvement in accuracy is
limited by the purity (98-99%) of the commercially available monomers used. Second,

5   all monomers were weighed into vials instead of being dispensed volumetrically.
Discrepancies between actual and reported monomer densities were found to be non-
negligible in some cases, leading to inaccuracies in dispensed mass. Third,
polymerizations were performed in the absence of solvent to maximize monomer
concentration, thus favoring the intermolecular addition reaction over the

10   intramolecular cyclization reaction. Eliminating the solvent also provides the added
benefits of increasing the reaction rate and obviating the solvent removal step. Finally,
since the previously employed methylene chloride solvent was not used, the reaction
temperature could be increased from 45 °C to 100 °C. Increasing temperature resulted
in an increased reaction rate and a decrease in the viscosity of the reacting mixture,

15   helping to offset the higher viscosity of the solvent-free system. The combined effect
of increased monomer concentration and reaction temperature resulted in a decrease in
reaction time from roughly 5 days to 5 hours.

We synthesized polymers Poly-1 and Poly-2 by adding 1,4-butanediol
diacrylate and 1,6-hexanediol diacrylate, respectively, to 1-amino butanol. Twelve

20   unique versions of each polymer were synthesized by varying amine/diacrylate mole
ratios between 0.6 and 1.4.

25
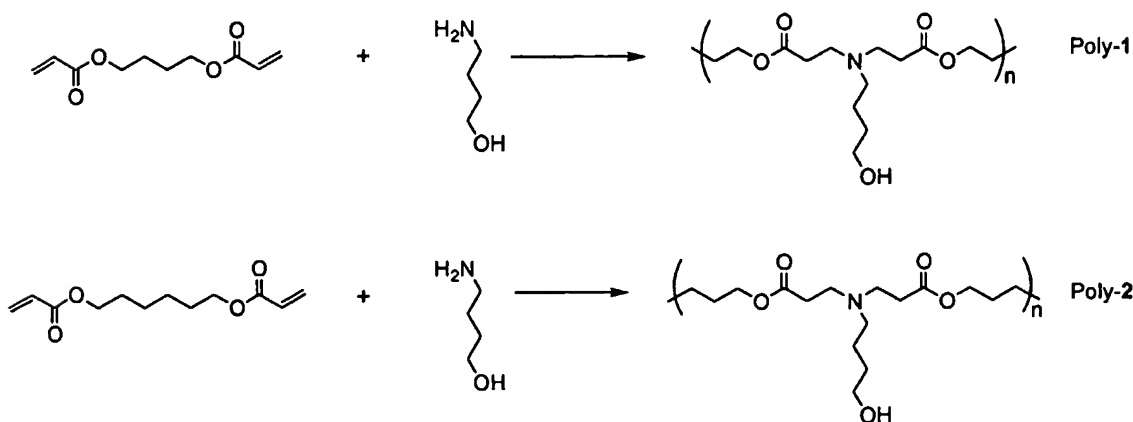
For both sets of polymers (Poly-1 and Poly-2), 7 of the 12 had amine/diacrylate ratios >
1, resulting in amine-terminated polymers, and 5 of the 12 had amine/diacrylate ratios <
1, resulting in acrylate-terminated polymers. After 5 hr reaction at 100 °C, polymers
were obtained as clear, slightly yellowish, viscous liquids. The polymers had

5   observable differences in viscosity, corresponding to differences in molecular weight.
Polymers were analyzed by organic phase gel permeation chromatography (GPC)
employing 70% THF/30% DMSO (v/v) + 0.1 M piperidine eluent. Polymer molecular
weights (M_w) ranged from 3350 (Poly-1, amine/diacrylate = 1.38) to 18,000 (Poly-1,
amine/diacrylate = 0.95), relative to polystyrene standards (Figure 16). Molecular

10   weight distributions were monomodal with polydispersity indices (PDIs) ranging from
1.55 to 2.20.

Luciferase Transfection Results. Transfection experiments were performed with all 24
synthesized polymers (12 each of Poly-1 and Poly-2) at 9 different polymer/DNA ratios

15   to determine the impact of molecular weight, polymer/DNA ratio, and chain end-group
on transfection efficiency (Figures 17 and 18). As a model system, we used the COS-7
cell line and a plasmid coding for the firefly luciferase reporter gene (pCMV-Luc) (600
ng/well). To facilitate performance of the nearly 1000 transfections (data obtained in
quadruplicate), experiments were done in 96-well plate format. Reporter protein

20   expression levels were determined using a commercially available luciferase assay kit
and a 96-well luminescence plate reader.

The data displayed in Figures 17 and 18 demonstrate that polymer molecular
weight, polymer/DNA ratio, and chain end-group impact the transfection properties of
both Poly-1 and Poly-2 polymers. One striking, and somewhat unexpected, result was

25   that none of the acrylate-terminated polymers mediated appreciable levels of
transfection under any of the evaluated conditions. This result may be more broadly
applicable for poly(β-amino esters), as we have yet to synthesize a polymer, using an
excess of diacrylate monomer, that mediates appreciable reporter gene expression at
any of the polymer/DNA ratios we have employed. These findings suggest that

30   perhaps only amine-terminated poly(β-amino esters) are suitable for use as gene
delivery vehicles. In contrast, there were distinct regions of transfection activity in the
MW–polymer/DNA space for amine-terminated versions of both Poly-1 and Poly-2

(Figures 17-A and 18-A). Maximal reporter gene expression levels of 60 ng luc/well and 26 ng luc/well were achieved using Poly-1 (M_w = 13,100) and Poly-2 (M_w = 13,400), respectively. These results compare quite favorably with PEI (polymer/DNA = 1:1 w/w), which mediated an expression level of 6 ng luc/well (data not shown)

5   under the same conditions.

While the highest levels of transfection occurred using the higher molecular weight versions of both polymer structures, the optimal polymer/DNA ratios for these polymers were markedly different (polymer/DNA = 150 for Poly-1, polymer/DNA = 30 for Poly-2). The transfection results we have obtained for Poly-1 and Poly-2

10   highlight the importance of optimizing polymer molecular weight and polymer/DNA ratio, and the importance of controlling chain end-groups. Further, the fact that two such similar polymer structures, differing by only two carbons in the repeat unit, have such different optimal transfection parameters emphasizes the need to perform these optimizations for each unique polymer structure. To improve our understanding of the

15   obtained transfection results, we chose to study two important delivery characteristics that directly impact transgene expression, cytotoxicity and the ability to enter cells *via* endocytosis (Wiethoff, C.M. and C.R. Middaugh, *Barriers to nonviral gene delivery.* Journal of Pharmaceutical Sciences, 2003. 92(2): p. 203-217; incorporated herein by reference).

20

Cytotoxicity. We evaluated the cytotoxicities of the various polymer/DNA complexes using a standard MTT/thiazolyl blue dye reduction assay. The experiments were performed exactly as the transfection experiments described above, except that instead of assaying for reporter gene expression on day 3, the MTT assay was performed after

25   day 1 (see Materials and Methods). We initially hypothesized that the lack of transfection activity observed for acrylate-terminated polymers may have been due to the cytotoxicity of the acrylate end-groups. Figures 19-B and 20-B do indicate that high concentrations of acrylate are cytotoxic, as viability is seen to decrease with increasing polymer/DNA ratio and decreasing polymer M_w (lower M_w corresponds to a

30   higher concentration of end-groups). However, cytotoxicity of acrylates does not sufficiently explain the lack of transfection activity at lower polymer/DNA ratios or higher molecular weights. Data shown in Figure 19-A demonstrates that cytotoxicity is

not a limiting factor for Poly-1 vectors, since cells remain viable even at the highest polymer/DNA ratios. On the other hand, the data displayed in Figure 20-A suggests that cytotoxicity is a major factor limiting the transfection efficiency of Poly-2 vectors, especially for the lower molecular weight polymers. This result may explain why

5   transfection activity is non-existent or decreasing for Poly-2 vectors at polymer/DNA > 30 (see Figure 18-A).

Cellular Uptake. The ability of polymer/DNA complexes to be taken up by cells was evaluated using a previously described flow cytometry-based technique to measure the

10   fluorescence of vector-delivered DNA (Akinc, A., et al., *Parallel synthesis and biophysical characterization of a degradable polymer library of gene delivery.* J. Am. Chem. Soc., 2003; incorporated herein by reference). Briefly, polymer/DNA complexes were prepared using plasmid DNA covalently labeled with the fluorescent dye Cy5. To allow for comparison of the cellular uptake data with the gene expression

15   data outlined above, complexes were formed at the same polymer/DNA ratios and in the same manner as in the transfection experiments. Labeled complexes were incubated with COS-7 cells for 1 hr at 37 °C to allow for uptake. The relative level of particle uptake was then quantified by measuring the fluorescence of cells loaded with Cy5-labeled DNA. The results of these uptake experiments are summarized in Figures

20   21 and 22. Data shown in Figure 21-B and 22-B suggest that the lack of transfection activity for the acrylate-terminated polymers is not due to cytotoxicity, as initially thought, but rather to an inability to enter the cell. Similarly, Poly-1 complexes are severely uptake-limited at all but the highest polymer/DNA ratios (Figure 21-A). While this data doesn't correlate exactly with the transfection results obtained for Poly-

25   1, it is consistent with the fact that transfection activity is not observed until very high polymer/DNA ratios are employed. Poly-2 complexes show no appreciable cellular uptake at polymer/DNA ratios < 30 and increasing levels of uptake as polymer/DNA ratios increase beyond 30 (Figure 22-A). This result, combined with the above cytotoxicity results, helps to explain the transfection activity of Poly-2 complexes. At

30   polymer/DNA ratios less than 30, complexes do not effectively enter the cell, but as polymer/DNA ratios increase much beyond 30, cytotoxicity begins to limit transfection efficiency, resulting in optimal transfection activity near polymer/DNA = 30.

Where endocytosis is the main route of cellular entry, the effective formation of nanometer-scale polymer/DNA complexes is one requirement for achieving high levels of cellular uptake (De Smedt, S.C., J. Demeester, and W.E. Hennink, *Cationic polymer based gene delivery systems.* Pharmaceutical Research, 2000. 17(2): p. 113-126;

5   Prabha, S., et al., *Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles.* International Journal of Pharmaceutics, 2002. 244(1-2): p. 105-115; each of which is incorporated herein by reference). The poor uptake levels observed for many of the polymer/DNA complexes may have been attributable to the unsuccessful formation of stable, nanoscale complexes. Unfortunately, the poor

10   neutral pH solubility of the polymers prevented making dynamic light scattering measurements of complex size using the transfection medium (Opti-MEM reduced serum media, pH 7.2) as the diluent. The obtained readings were attributable to polymer precipitates, which were in some cases visible as turbidity in solutions of polymer in Opti-MEM. However, we were able to measure the effective diameters of

15   complexes using 25 mM sodium acetate (pH 5) as the sample diluent. While this data cannot shed light on the size or stability of complexes in the transfection medium, it can indicate whether complexes were successfully formed prior to addition to the transfection medium. Specifically, we found that the lower molecular weight versions ($M_w < 10,700$) of Poly-1 were unable to form nanoscale complexes, even in acetate

20   buffer. In all other cases we found that nanometer-sized polymer/DNA complexes were formed. While these results may explain the poor uptake levels associated with low molecular weight versions of Poly-1, they do not satisfactorily explain the low uptake activity of the acrylate-terminated polymers or the dependence of polymer/DNA ratio on cellular uptake. Although particle size and stability are important factors

25   impacting cellular uptake, it is likely that other, yet unidentified, factors must also be considered in order to provide a more complete explanation of the obtained cellular uptake results.

Enhancement of Transfection Using a Co-Complexing Agent. Both Poly-1 and Poly-2

30   require relatively high polymer/DNA weight ratios to achieve high levels of gene transfer. One explanation may be that, compared to other polymers often used to compact DNA (*e.g.,* polylysine (PLL) and PEI), these polymers have relatively low

nitrogen densities. Poly-1 has a molecular weight per nitrogen atom (MW/N) of 301, and Poly-2 has a MW/N of 329. By comparison, for PLL and PEI, these figures are roughly 65 and 43, respectively. It might be possible to reduce the amount of Poly-1 or Poly-2 necessary to achieve high levels of transfection by incorporating a small amount

5   of co-complexing agent. This approach could be especially beneficial for Poly-2 vectors, since cytotoxicity appears to be an important limitation for these vectors. To test this hypothesis, PLL and PEI were used as model co-complexing agents. We focused our attention on the most promising member in each of the Poly-1 (amine-terminated, M_w = 13,100) and Poly-2 (amine-terminated, M_w = 13,400) sets of

10   polymers. The data displayed in Figures 23 and 24 indicate that a significant reduction in polymer could be achieved, while maintaining high levels of transfection efficiency, through the use of PLL or PEI as co-complexing agents. In some cases, significant enhancement of transfection activity was realized. As expected, this co-complexation approach was particularly beneficial for Poly-2 vectors. This work, and prior work

15   (Wagner, E., et al., *Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle.* Proc. Natl. Acad. Sci. U. S. A., 1992. 89(17): p. 7934-8; Fritz, J.D., et al., *Gene transfer into mammalian cells using histone-condensed plasmid DNA.* Hum Gene Ther, 1996. 7(12): p. 1395-404; Pack, D.W., D. Putnam, and

20   R. Langer, *Design of imidazole-containing endosomolytic biopolymers for gene delivery.* Biotechnol. Bioeng., 2000. 67(2): p. 217-23; Lim, Y.B., et al., *Self-Assembled Ternary Complex of Cationic Dendrimer, Cucurbituril, and DNA: Noncovalent Strategy in Developing a Gene Delivery Carrier.* Bioconjug Chem, 2002. 13(6): p. 1181-5; each of which is incorporated herein by reference), demonstrates that the

25   blending of polymers with complementary gene transfer characteristics can in some cases produce more effective gene delivery reagents.

GFP Transfections

       To further evaluate the transfection properties of the Poly-1/PLL and Poly-

30   2/PLL blended reagents, we performed transfection experiments using a reporter plasmid coding for green fluorescent protein (pCMV-EGFP). Though the luciferase and GFP transfection assay systems both measure transfection activity, they provide

different types of information. The luciferase system quantifies the total amount of exogenous luciferase protein produced by all the cells in a given well, providing a measure of cumulative transfection activity. In contrast, the GFP system can be used to quantify the percentage of cells that have been transfected, providing a cell-by-cell

5   measure of transfection activity. Both systems are useful and offer complementary information regarding the transfection properties of a given gene delivery system.

GFP transfections were performed in a similar manner as the luciferase experiments, but were scaled up to 6-well plate format (5 μg plasmid/well). COS-7 cells were transfected using Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1 w/w/w) and

10   Poly-2/PLL (Poly-2:PLL:DNA = 15:0.4:1 w/w/w). Lipofectamine 2000 (μL reagent: μg DNA = 1:1), PEI (PEI:DNA = 1:1 w/w, N/P ~ 8), and naked DNA were used as controls in the experiment. After 1 hr incubation with cells, vectors were removed and fresh growth media was added. Two days later GFP expression was assayed using a flow cytometer. Nearly all cells transfected with Poly-1/PLL were

15   positive for GFP expression (Figures 25 and 26). Experiments indicated that Poly-2/PLL vectors were less effective, resulting in roughly 25% positive cells. Positive controls Lipofectamine 2000 and PEI were also able to mediate effective transfection of COS-7 cells under the conditions employed. Although Lipofectamine 2000 and PEI transfections resulted in nearly the same percentage of GFP-positive cells as Poly-

20   1/PLL, the fluorescence level of GFP-positive cells was higher for Poly-1/PLL (mean fluorescence = 6033) than that of both Lipofectamine 2000 (mean fluorescence = 5453) and PEI (mean fluorescence = 2882). Multiplying the percentage of positive cells by the mean fluorescence level of positive cells provides a measure of aggregate expression for the sample and should, in theory, better correlate with the results of

25   luciferase gene expression experiments. Quantifying total GFP expression in this manner indicates that the highest expression level is achieved by Poly-1/PLL, followed by Lipofectamine 2000 and PEI. This result is in general agreement with the luciferase expression results.

Experiments have shown that Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1

30   w/w/w) is a highly effective vector for transfecting COS-7 cells. The ability of this vector to mediate transfection in three other commonly used cell lines (CHO, NIH 3T3, and HepG2) was also investigated. It is very likely that each of these cell lines have

optimal transfection conditions that differ from those used to transfect COS-7 cells;
however, as a preliminary evaluation of the ability to transfect multiple cell lines,
transfections were performed in the same manner and under the same conditions as the
COS-7 transfections. Results indicate that Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1

5   w/w/w) is able to successfully transfect CHO, NIH 3T3, and HepG2 cells, though not
as effectively as COS-7 cells (Figure 27). This is not too surprising since the vector
used was optimized by screening for gene transfer in COS-7 cells. Optimization of
vector composition and transfection conditions specific for each cell type would be
expected to result in even higher transfection levels.

10

Summary

In this work, the role of polymer molecular weight, polymer chain end-group,
and polymer/DNA ratio on a number of important gene transfer properties has been
investigated. All three factors were found to have a significant impact on gene transfer,

15   highlighting the benefit of carefully controlling and optimizing these parameters. In
addition, the incorporation of a small amount of PLL, used to aid complexation, further
enhances gene transfer. Through these approaches degradable poly(beta-amino esters)-
based vectors that rival some of the best available non-viral vectors for *in vitro* gene
transfer.

20

Example 6-Further Studies of Selected poly(beta-amino esters)

To further characterize and synthesize some of the poly(beta-amino esters)
identified in previous screens, a twenty-one polymers were re-synthesized at various

25   ratios of amine monomer to acrylate monomer. The resulting polymers were
characterized by gel permeation chromatography to determine the molecular weight
and polydispersitie of each polymer. Each of the polymer was then tested for its ability
to transfect cells.

30   **Polymer Synthesis.** The polymers were synthesized as described in Example 5.
Several versions of each polymer were created by varying the amin/diacrylated
stoichiometric ratio. For example, C36-1 corresponds to the stoichiometric ratio of 1.4,

and C36-12 to 0.6, with all the intermediates given in the table below:

Version of C36	Amine:Acrylate Stoichiometric Ratio
C36-1	1.4
C36-2	1.3
C36-3	1.2
C36-4	1.1
C36-5	1.05
C36-6	1.025
C36-7	1.0
C36-8	0.975
C36-9	0.950
C36-10	0.9
C36-11	0.8
C36-12	0.6

The polymers were typically prepared in glass vials with no solvent at 100 °C for 5 hours. In some syntheses, the polymerization at 100 °C yielded highly cross-linked polymers when certain monomers such as amine 94 were used; therefore, the polymerization reactions were repeated at 50 °C with 2 mL of DMSO added to avoid cross-linking.

The resulting polymers were analyzed by GPC as described in Example 5. The molecular weights and polydispersities of each of the polymers is shown in the table below:

Polymer	M_w	M_n	Polydispersity
F28-1	5540	2210	2.50678733
F28-2	6150	2460	2.5
F28-3	8310	2920	2.845890411
F28-4	11600	3660	3.169398907
F28-5	16800	4360	3.853211009
F28-6	16100	4850	3.319587629
F28-7	18000	5040	3.571428571
F28-8	18200	5710	3.187390543
F28-9	22300	7880	2.829949239
F28-10	23700	8780	2.699316629
F28-11	12100	5660	2.137809187
F28-12	4850	2920	1.660958904
C36-1	7080	3270	2.165137615
C36-2	5100	2640	1.931818182

C36-3	21200	8090	2.620519159
C36-4	20500	6710	3.05514158
C36-5	112200	33200	3.379518072
C36-6	21700	6890	3.149492017
C36-7	36800	15700	2.343949045
C36-8	35700	12600	2.833333333
C36-9	35200	15100	2.331125828
C36-10	22500	9890	2.275025278
C36-11	26000	6060	4.290429043
D60-1	1890	1400	1.35
D60-2	2050	1520	1.348684211
D60-3	2670	1720	1.552325581
D60-4	3930	2210	1.778280543
D60-5	5130	2710	1.89298893
D60-6	5260	2800	1.878571429
D60-7	1130	1090	1.036697248
D60-8	1840	1510	1.218543046
D60-9	6680	3440	1.941860465
D60-10	8710	4410	1.975056689
D60-11	9680	4410	2.195011338
D60-12	7450	3470	2.146974063
D61-1	1710	1410	1.212765957
D61-2	2600	1790	1.452513966
D61-3	3680	2280	1.614035088
D61-4	4630	2550	1.815686275
D61-5	NA	NA	NA
D61-6	NA	NA	NA
D61-7	6110	3250	1.88
D61-8	6410	3190	2.009404389
D61-9	6790	3440	1.973837209
D61-10	8900	4350	2.045977011
D61-11	10700	4600	2.326086957
D61-12	6760	2900	2.331034483
F32-1	10300	3260	3.159509202
F32-2	11100	3490	3.180515759
F32-3	16600	4820	3.443983402
F32-4	17300	5390	3.209647495
F32-5	18600	5830	3.190394511
F32-6	26200	8290	3.160434258
C32-1	6670	2810	2.37366548
C32-2	18100	5680	3.186619718
C32-3	19300	6060	3.184818482

C32-4	25600	9100	2.813186813
C32-5	25000	7860	3.180661578
C32-6	25700	8440	3.045023697
C86-1	14200	2900	4.896551724
C86-2	21000	2900	7.24137931
C86-3	27500	4590	5.991285403
U94-1	10700	3530	3.031161473
U94-2	NA	NA	NA
U94-3	NA	NA	NA
F32-1	10300	3260	3.159509202
F32-2	11100	3490	3.180515759
F32-3	16600	4820	3.443983402
F32-4	17300	5390	3.209647495
F32-5	25000	7860	3.180661578
F32-6	26200	8290	3.160434258
C32-1	6670	2810	2.37366548
C32-2	18100	5680	3.186619718
C32-3	19300	6060	3.184818482
C32-4	25600	9100	2.813186813
C32-5	25000	7860	3.180661578
C32-6	25700	8440	3.045023697
U86-1	Unusual	NA	NA
U86-2	Unusual	NA	NA
U86-3	Unusual	NA	NA
U86-4	Unusual	NA	NA
U85-5	Unusual	NA	NA
JJ32-1	9730	4010	2.426433915
JJ32-2	12100	4580	2.641921397
JJ32-3	19400	6510	2.980030722
JJ32-4	27900	10000	2.79
JJ32-5	32600	9720	3.353909465
JJ32-6	28900	9870	2.928064843
JJ36-1	7540	3550	2.123943662
JJ36-2	143500	59600	2.407718121
JJ36-3	20100	7310	2.749658003
JJ36-4	30200	10200	2.960784314
JJ36-5	33900	10600	3.198113208
JJ36-6	36100	12500	2.888
JJ28-1	7550	3240	2.330246914
JJ28-2	9490	3460	2.742774566
JJ28-3	16800	5420	3.099630996
JJ28-4	23300	8090	2.880098888

JJ28-5	25500	7700	3.311688312
JJ28-6	32100	10900	2.944954128
U28-1	7190	2580	2.786821705
U28-2	10700	3990	2.681704261
U28-3	15600	7300	2.136986301
U28-4	20400	9880	2.064777328
U28-5	20500	9670	2.119958635
U28-6	24200	13000	1.861538462
E28-1	5900	3280	1.798780488
E28-2	7950	3550	2.23943662
E28-3	14300	6300	2.26984127
E28-4	6990	3320	2.105421687
E28-5	17400	8180	2.127139364
E28-6	19300	9030	2.137320044
LL6-1	2380	1570	1.515923567
LL6-2	3350	2070	1.618357488
LL6-3	4110	2340	1.756410256
LL6-4	5750	3010	1.910299003
LL6-5	7810	5050	1.546534653
LL6-6	6950	4190	1.658711217
LL8-1	3160	1910	1.654450262
LL8-2	3630	2560	1.41796875
LL8-3	5300	3520	1.505681818
LL8-4	6000	3320	1.807228916
LL8-5	8160	4730	1.725158562
LL8-6	7190	4650	1.546236559
U36-1	7290	3370	2.163204748
U36-2	11100	5000	2.22
U36-3	12600	5470	2.303473492
U36-4	21500	8550	2.514619883
U36-5	24700	9430	2.619300106
U36-6	31700	10700	2.962616822
E36-1	6030	3130	1.926517572
E36-2	8510	4040	2.106435644
E36-3	12800	5730	2.233856894
E36-4	18200	7620	2.388451444
E36-5	20100	8050	2.49689441
E36-6	32900	10900	3.018348624
U32-1	9830	3790	2.593667546
U32-2	12000	4460	2.69058296
U32-3	18200	6780	2.684365782
U32-4	25200	11100	2.27027027

U32-5	26500	9360	2.831196581
U32-6	26200	10600	2.471698113
E32-1	7070	3310	2.135951662
E32-2	9920	4180	2.373205742
E32-3	14700	6080	2.417763158
E32-4	23500	9160	2.565502183
E32-5	28800	10000	2.88
E32-6	26900	10300	2.611650485
C94-1	6760	3110	2.173633441
C94-2	10800	4190	2.577565632
C94-3	18000	5330	3.377110694
C94-4	38900	6660	5.840840841
C94-5	Didn't dissolve	NA	NA
C94-6	Didn't dissolve	NA	NA
D94-1	6030	2980	2.023489933
D94-2	6620	3370	1.964391691
D94-3	9680	3950	2.450632911
D94-4	11500	4510	2.549889135
D94-5	13700	4940	2.773279352
D94-6	18800	5650	3.327433628
F94-1	5570	2740	2.032846715
F94-2	7670	3180	2.411949686
F94-3	12600	4230	2.978723404
F94-4	20300	5160	3.934108527
F94-5	21500	5390	3.988868275
F94-6	27300	6310	4.326465927
JJ94-1	7750	3360	2.306547619
JJ94-2	12700	4590	2.766884532
JJ94-3	30500	7280	4.18956044
JJ94-4	Didn't dissolve	NA	NA
JJ94-5	Didn't dissolve	NA	NA
JJ94-6	Didn't dissolve	NA	NA
F86-1	3940	2630	1.498098859
F86-2	5300	3190	1.661442006
F86-3	7790	4040	1.928217822
F86-4	11000	5410	2.033271719
F86-5	10600	5650	1.876106195
F86-6	13300	6440	2.065217391
D86-1	4610	2830	1.628975265
D86-2	5570	3290	1.693009119
D86-3	7120	3770	1.888594164
D86-4	8310	4440	1.871621622

D86-5	8950	4710	1.900212314
D86-6	10400	5010	2.075848303
U86-1	5940	3500	1.697142857
U86-2	7780	4430	1.756207675
U86-3	11900	6540	1.819571865
U86-4	15100	7630	1.979030144
U86-5	16300	8950	1.82122905
U86-6	18100	9810	1.845056065
E86-1	4880	3140	1.554140127
E86-2	6300	3790	1.662269129
E86-3	9780	5140	1.902723735
E86-4	12500	6350	1.968503937
E86-5	13400	6820	1.964809384
E86-6	15500	7280	2.129120879
JJ86-1	5460	3370	1.620178042
JJ86-2	6880	4080	1.68627451
JJ86-3	11900	6180	1.925566343
JJ86-4	14200	7000	2.028571429
JJ86-5	20500	9090	2.255225523
JJ86-6	16300	7770	2.097812098
C86-1	4870	3030	1.607260726
C86-2	5720	3460	1.653179191
C86-3	9970	5060	1.970355731
C86-4	14200	7000	2.028571429
C86-5	17700	8500	2.082352941
C86-6	17800	8500	2.094117647
C80-1	2450	1790	1.368715084
C80-2	3770	2370	1.5907173
C80-3	6080	3370	1.804154303
C80-4	7960	4310	1.846867749
C80-5	9030	4660	1.93776824
C80-6	12600	6050	2.082644628
E80-1	2840	2010	1.412935323
E80-2	3720	2420	1.537190083
E80-3	6080	3650	1.665753425
E80-4	7210	4240	1.700471698
E80-5	7640	4290	1.780885781
E80-6	9000	5310	1.694915254
JJ80-1	3410	2180	1.564220183
JJ80-2	4590	2890	1.588235294
JJ80-3	8430	4750	1.774736842
JJ80-4	11300	6560	1.722560976

JJ80-5	13200	7160	1.843575419
JJ80-6	11600	6540	1.773700306
U80-1	4300	2680	1.604477612
U80-2	5130	3020	1.698675497
U80-3	8320	4700	1.770212766
U80-4	9130	4880	1.870901639
U80-5	11300	5750	1.965217391
U80-6	11200	5920	1.891891892

Luciferase Transfection Assay. As described in Example 5, COS-7 cells were transfected with pCMV-Luc DNA using each of the polymers at polymer-to-DNA ratios ranging from 10:1 to 100:1 (weight:weight). Luciferase expresseion was

5 analysed using Bright-Glo assay kits (Promega). Luminescence was measured for each transfection, and the luminescence was used to calculate nanograms of Luciferase enzyme as described in Example 5. Experiments were done in quadruplicate, and the values shown in the tables below are the averaged values from the four experiments. These data are shown below for each polymer synthesized.

10

C36-1	C36-2	C36-3	C36-4	C36-5	C36-6	Ratio
0.168527	0.345149	0.627992	0.152258	0.068355	0.094073	10
3.58467	0.12639	21.27867	2.145388	0.163042	0.184298	20
4.295966	0.927605	18.84046	4.750661	0.287989	1.063834	30
7.150343	1.137242	17.04771	7.529555	0.080757	0.332789	40
3.74705	1.180274	6.875879	9.710764	0.582186	1.963895	60
0.705683	0.212297	0.560245	7.221382	5.003849	5.813189	100

C36-7	C36-8	C36-9	C36-10	C36-11	C-36-12	Ratio
0.164373	0.085336	0.116502	0.042173	0.062905	0.18877	10
0.134132	0.096043	0.091152	0.032851	0.032115	0.383965	20
0.020768	0.021203	0.0665	0.021953	0.017807	0.288102	30
0.05027	0.060731	0.017768	0.011885	0.008923	0.128469	40
0.031233	0.048807	0.025626	0.012516	0.002606	0.213173	60
0.116587	0.129504	0.332497	0.123413	0.058442	0.250708	100

C86-1	C86-2	C86-3	Ratio
0.157713	0.475708	1.093272	10
0.242481	0.616621	1.439904	20
0.396888	0.992601	1.758045	30
0.300173	1.276707	1.901677	40

D60-1	D60-2	D60-3	D60-4	D60-5	D60-6	Ratio

0.604984	0.443875	0.363271	0.260475	0.498462	0.466087	10
0.115174	0.174976	0.250613	0.40783	0.587186	0.89381	20
0.138372	0.45915	0.81101	0.773161	1.264634	1.438474	30
0.135287	0.506303	2.344053	1.695591	2.302305	2.959638	40
0.203804	0.679718	3.908348	2.216808	3.129304	4.335511	60
0.233546	0.640246	0.251146	3.112999	7.65786	6.759895	100

D60-7	D60-8	D60-9	D60-10	D60-11	D60-12	Ratio
0.299777	0.333863	0.434027	0.46862	0.387458	0.211083	10
0.237477	0.266398	1.211246	1.385232	1.034892	0.215027	20
0.339709	0.665539	2.958346	5.607664	3.514454	0.485295	30
0.499842	1.216181	4.406196	6.736276	5.121445	0.444359	40
1.297394	1.009228	5.951785	9.565956	7.193687	0.35831	60
5.399266	0.135852	5.725666	10.45568	5.414051	0.245279	100

D61-1	D61-2	D61-3	D61-4	D61-5	D61-6	Ratio
0.329886	0.29803	0.190101	0.142813	0.114565	0.227593	10
0.299409	0.710035	0.295508	0.288845	0.247909	0.32839	20
0.155568	0.680763	0.618022	0.651633	0.402721	1.831437	30
0.085824	0.620294	3.722971	4.572264	3.010274	11.69027	40
0.188357	0.187979	3.970054	7.147033	10.85674	9.238981	60
0.019321	0.001369	0.034958	0.033062	0.202601	0.131544	100

D61-7	D61-8	D61-9	D61-10	D61-11	D61-12	Ratio
0.153122	0.180646	0.1073	0.244713	0.231561	0.18571	10
0.203312	0.217288	0.191108	0.185759	0.270723	0.119897	20
0.539455	0.239807	0.140418	0.174014	0.320869	0.094186	30
1.679507	1.020126	0.584908	0.229946	0.474142	0.154025	40
12.69543	5.9829	7.008946	1.308281	0.301803	0.067526	60
1.271189	2.402989	3.186707	5.576734	1.343239	0.115366	100

U94-1	U94-2	U94-3	Ratio
0.233894	0.127165	0.804911	10
0.179855	1.35532	13.53974	20
0.275078	16.26098	20.65427	30
1.161574	19.93922	13.08098	40
1.961067	18.39299	9.319949	60
13.0485	7.591092	1.647718	100

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C32-1	C32-2	C32-3	C32-4	C32-5	C32-6	Ratio
0.137436	0.544141	0.138034	0.112832	0.087552	0.131699	10
0.159782	28.93062	14.3276	0.316178	0.125792	0.242881	20
0.166661	53.90695	24.83791	0.67551	0.193545	0.181321	30
0.392402	90.62006	49.11244	2.271509	0.563168	0.632798	40
6.034825	73.59378	46.31	2.490156	0.111248	0.273411	60

| 38.17463 | 60.21433 | 51.86994 | 16.43407 | 2.01284 | 2.619288 | 100 |

F32-1	F32-2	F32-3	F32-4	F32-5	F32-6	Ratio
0.746563	2.446604	1.288067	0.210478	0.202798	0.112283	10
20.84138	20.94165	11.46963	1.780569	10.90572	0.100889	20
23.8042	23.7095	13.34488	5.01115	9.510119	0.255589	30
17.47681	17.35353	14.74619	8.361793	6.436393	0.599084	40
10.54807	11.78762	13.58168	7.499322	4.865577	0.322946	60
0.072034	0.090408	0.332458	2.300951	2.434663	1.644695	100

F28-1	F28-2	F28-3	F28-4	F28-5	F28-6	Ratio
0.245612	0.247492	0.140455	0.203674	0.09426	0.131075	10
0.464885	0.192584	0.217777	0.213391	0.171565	0.397716	20
0.290643	0.19239	0.396845	0.433955	0.361789	2.02073	30
0.325066	0.189405	1.048323	2.088649	3.888705	19.95507	40
0.108766	0.164709	13.95859	0.411927	7.851029	21.77709	60
0.163978	6.619239	6.832291	8.409421	6.682506	2.958283	100

F28-7	F28-8	F28-9	F28-10	F28-11	F28-12	Ratio
0.094505	0.043474	0.05224	0.050384	0.104016	0.149513	10
0.173987	0.098512	0.069287	0.057704	0.131067	0.028219	20
0.705917	0.254424	0.083005	0.04454	0.133842	0.001619	30
2.860034	0.928959	0.226468	0.076503	0.095093	0.003896	40
7.786755	1.932506	0.42898	0.028744	0.063298	0.001342	60
8.655579	12.729	1.396803	0.281853	0.115513	0.001145	100

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JJ28-1	JJ28-2	JJ28-3	JJ28-4	JJ28-5	JJ28-6	Ratio
0.122351	0.056864	0.030798	0.041065	0.02373	0.051849	10
0.060598	0.059073	22.46575	2.229717	0.076134	0.053754	20
0.174243	0.211589	21.92396	4.089533	0.121043	0.115906	30
0.133603	36.42899	69.60415	19.16868	0.292215	0.337099	40
1.011778	64.69601	71.50927	47.49171	0.755335	0.654333	60
60.46546	56.7025	53.44758	39.13032	25.81403	3.936471	100

JJ36-1	JJ36-2	JJ36-3	JJ36-4	JJ36-5	JJ36-6	Ratio
0.506359	1.634649	0.146132	0.053268	0.035023	0.033605	10
2.185596	4.332834	0.677853	0.017846	0.010687	0.004264	20
2.339652	5.039758	0.773873	0.024164	0.06932	0.009318	30
0.681878	1.871844	1.539743	0.087428	0.017886	0.009752	40
0.521703	1.592328	2.000554	0.201203	0.027165	0.004975	60
0.003277	0.067895	1.031285	0.284902	0.159879	0.008844	100

JJ32-1	JJ32-2	JJ32-3	JJ32-4	JJ32-5	JJ32-6	Ratio
0.392821	1.158486	0.191533	0.127891	0.099083	0.076569	10
17.51289	21.24103	1.803172	0.065286	0.160362	0.108887	20
38.08705	43.77517	24.76927	0.09612	0.063692	0.044659	30
25.9567	34.88211	26.36994	0.201907	0.015214	0.026165	40
11.37519	17.48944	29.59326	0.175101	0.052321	0.098545	60
1.3311	1.288845	6.86144	0.70071	0.178921	0.70361	100

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LL6-1	LL6-2	LL6-3	LL6-4	LL6-5	LL6-6	Ratio
0.405305	0.583416	0.520853	0.50183	0.656802	1.060478	10
0.411758	0.747731	0.460075	0.287671	0.382454	1.099616	20
0.809416	0.377302	1.253481	1.099976	2.59164	1.138122	30
0.475903	0.854576	1.812577	2.018906	2.837056	0.69298	40
0.139647	0.29034	0.939013	1.992525	2.250511	3.059824	60
0.00154	0.002408	0.223682	2.932931	3.939451	6.879564	100

LL8-1	LL8-2	LL8-3	LL8-4	LL8-5	LL8-6	Ratio
0.533009	1.180815	1.581011	2.254195	1.73015	1.76882	10
1.174539	1.228513	1.002632	2.369943	1.958308	2.928439	20
1.182611	1.620962	3.771897	3.988759	3.936124	0.000474	30
1.366191	1.875091	4.594308	4.253834	4.07168	0.000948	40
0.120086	0.866135	1.925861	4.423822	4.081074	5.083137	60
0.003316	0.029499	0.336777	4.077347	4.416413	4.241522	100

U28-1	U28-2	U28-3	U28-4	U28-5	U28-6	Ratio
0.049477	0.044465	0.045254	0.034669	0.031628	0.025942	10
0.111915	0.050661	0.03988	0.015399	0.049004	0.020729	20
0.041895	0.050582	0.048212	0.064917	0.069067	0.02756	30
0.122271	0.078429	1.647405	0.488561	1.036216	0.058913	40
0.059982	0.051095	18.03734	5.718868	1.991446	0.176516	60
0.059585	44.91463	51.17075	34.01612	32.39362	3.488256	100

E28-1	E28-2	E28-3	E28-4	E28-5	E28-6	Ratio
0.109892	0.150078	0.630192	0.187585	0.311968	0.168724	10
0.088189	0.509429	0.589377	0.106743	0.70723	0.144608	20
1.672278	12.4166	21.41889	3.405963	8.337341	1.32881	30
5.658186	12.17055	13.3351	7.272109	17.92266	5.089686	40
6.016333	5.424512	5.549474	5.185252	15.62457	8.706483	60
1.146098	0.804227	0.592348	0.529551	1.752402	5.438448	100

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U36-1	U36-2	U36-3	U36-4	U36-5	U36-6	Ratio
0.158334	0.388325	0.255439	0.139618	0.069735	0.054654	10
0.281155	2.119615	0.34803	0.196109	0.111805	0.067406	20
0.968904	2.501669	3.74982	0.370814	0.103744	0.077397	30
0.559332	1.595139	4.650123	0.65127	0.078343	0.029797	40
0.565322	0.540675	4.182981	1.59494	0.250723	0.029297	60
0.238507	0.008686	1.052448	2.269889	1.310025	0.23654	100

E36-1	E36-2	E36-3	E36-4	E36-5	E36-6	Ratio
0.130066	2.940734	0.350723	0.077836	0.051293	0.018123	10
0.496911	1.866482	2.236257	0.135236	0.063655	0.020018	20
1.044698	0.617286	4.27308	0.882725	0.187495	0.01532	30
0.342085	0.025849	1.935655	1.170393	0.23494	0.004896	40
0.112427	0.211108	1.068847	1.362371	0.628612	0.070175	60
0.002566	0.003672	0.131476	1.367514	1.194649	0.11883	100

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U32-1	U32-2	U32-3	U32-4	U32-5	U32-6	Ratio
0.202681	0.107654	0.035536	0.037195	0.041027	0.047701	10
0.106511	0.084192	0.067327	0.012951	0.028982	0.012003	20
1.497135	2.867785	1.828273	0.056586	0.033682	0.010305	30
4.411592	13.8534	0.272404	0.056588	0.029377	0.021045	40
17.28347	38.37457	3.026925	0.017452	0.015556	0.060968	60
11.81885	13.6584	15.23297	0.673546	0.121004	0.15261	100

E32-1	E32-2	E32-3	E32-4	E32-5	E32-6	Ratio
0.481414	0.898699	0.149685	0.093788	0.086886	0.046001	10
5.170892	6.057429	1.52106	0.054373	0.099557	0.01378	20
0.965091	2.279423	4.380769	0.112159	0.027166	0.038894	30
0.848062	1.086906	3.971834	0.13767	0.068236	0.090555	40
0.225141	0.688091	2.653561	0.570809	0.080796	0.01603	60
0.046762	0.176583	0.897883	1.365759	0.845009	0.111133	100

C94-1	C94-2	C94-3	C94-4	Ratio
0.289113	0.086166	0.151651	0.203119	10
0.133487	0.045908	0.067867	7.650297	20
0.293536	0.086328	0.853319	10.31612	30
0.198737	0.170611	1.955433	9.745005	40
0.312808	0.908991	3.536115	3.580573	60
0.32801	0.853063	2.414853	1.731594	100

D94-1	D94-2	D94-3	D94-4	D94-5	D94-6	Ratio
0.223798	0.091225	9.083876	0.272732	0.46751	5.545365	10
9.682036	15.16589	24.65534	25.45656	27.30727	25.52283	20
14.53736	22.28715	29.68042	38.12112	42.28773	33.35092	30
9.804481	14.97104	18.63768	28.87773	35.67401	28.4263	40
6.36291	11.60176	16.02556	27.2195	29.006	16.62105	60
2.681942	2.585502	3.03267	9.218975	12.48001	9.63693	100

The following table shows the synthesis of the polymers listed on the right using ratios of amine monomer to acrylate monomer ranging from 1.4 to 0.6 as described above. Each synthesis of the polymer was then tested for Luciferase transfection using six different polymer to DNA ratios (each experiment was done in quadruplicate). The data in the table represents the Luciferase activity under the best polymer to DNA ratio.

Polymer	Mole Ratio of Amine Monomer to Acrylate Monomer											
	1.400	1.300	1.200	1.100	1.050	1.025	1.000	0.975	0.950	0.900	0.800	0.600
C36	1.18	7.150343	21.27867	9.710764	5.003849	5.813189	0.164373	0.129504	0.332497	0.123413	0.062905	0.383965
C86-1	0.396888	1.276707	1.901677									
D60-NoDMS	0.604984	0.679718	3.908348	3.112999	7.65786	6.759895	5.399266	1.216181	5.951785	10.45568	7.193687	0.485295
D61-NoDMSO	0.329886	0.710035	3.970054	7.147033	10.85674	11.69027	12.69543	5.9829	7.008946	5.576734	1.343239	0.18571
U94-1		13.0485	19.93922	20.65427								
F32		23.8042	23.7095	14.74619	8.361793	10.90572	1.644695					
F28	0.464885	6.619239	13.95859	8.409421	7.851029	21.77709	8.655579	12.729	1.396803	0.281853	0.133842	0.149513
JJ36		2.339652	5.039758	2.000554	0.284902	0.159879	0.033605					
JJ32		38.08705	43.77517	29.59326	0.70071	0.178921	0.70361					
LL6		0.809416	0.854576	1.812577	2.932931	3.939451	6.879564					
LL8		1.366191	1.875091	4.594308	4.423822	4.416413	5.083137					
U28		0.122271	44.91463	51.17075	34.01612	32.39362	3.488256					
E28		6.016333	12.4166	21.41889	7.272109	17.92266	8.706483					
U36		0.968904	2.501669	4.650123	2.269889	1.310025	0.23654					
E36		1.044698	2.940734	4.27308	1.367514	1.194649	0.11883					
U32		17.28347	38.37457	15.23297	0.673546	0.121004	0.15261					
E32		5.170892	6.057429	4.380769	1.365759	0.845009	0.111133					
C94		0.32801	0.908991	3.536115	10.31612	0.237264						
D94		14.53736	22.28715	29.68042	38.12112	42.28773	33.35092					
F94		0.490553	4.108079	3.530146	8.596197	8.200005	9.892867					
JJ94		1.906203	3.87783	11.99391	0.422621	0.590515	0.787162					
E28			8.572344	20.89276	0.290904	14.67007						
U28			0.221192	0.515945	0.537706	0.237264						
JJ86		0.503544	0.775429	0.794641	1.055779	1.491694	0.707425					
C86		0.159044	0.279389	0.637804		1.025192	1.422756					

	1	2	3	4	5	6	7	8	9	10	11	12
U86	0.435638	0.196901	0.124174	0.304979	0.193936	0.189116						
E86	0.27629	0.248666	3.633516	6.288459	9.202082	8.484492						
C80	0.578045	0.102763	0.111304	0.528832	0.887811	0.961981						
E80	1.697744	2.624376	3.868368	3.757709	4.412999	1.895016						
JJ80	0.5771	0.4786	2.199535	3.226941	3.173297	2.653163						
U80	0.119897	0.514079	2.658899	3.552453	4.456214	9.776965						
D24	0.526541	2.021508	0.324894	36.64054	0.244347	0.365902						
E24	0.098517	0.057019	7.404745	1.12791	0.329197	0.326384						
JJ24	0.199154	0.188046	0.090045	0.064921	0.085969	0.032536						
B17	0.72135	0.44715	0.996508	1.067002	1.098275	2.197846						
M17	0.580405	0.433646	0.484853	0.515332	0.672973	0.763891	1.360645	2.580579	2.016952	7.131925	2.21044	0.743141
M17-DMSO	0.550188	0.409299	0.304933	0.35066	0.404506	0.356775	0.392121	0.376042	0.34777	0.315241	0.468629	0.705352
B17 D	0.614141	0.505597	0.339801	0.319609	0.399828	0.406147						
II28	0.334806	0.137402	0.079535	0.165612	0.097702	0.126852						
II36	0.331156	0.344857	0.209068	0.107622	0.151587	0.170145						
II32	0.046949	0.026455	0.070335	0.047503	0.033368	0.0261						
C20	0.126573	0.098883	0.088576	1.003372	0.11883	0.138726						
JJ20	0.085338	40.19751	47.297	5.655104	0.142257	0.099295						
E20	18.94919	36.04742	31.97179	24.49526	21.63777	10.55303						
C25	0.11105	0.044342	0.06737	0.031668	0.088061	0.05876						
U25	0.055718	0.056074	0.069147	0.047859	0.04537	0.088747						
D25	0.105104	0.352229	3.212175	6.167455	1.617246	1.792186						
D70	8.351581	5.601802	9.168348	14.68248	6.364419	0.469952						
D60	7.304436	11.02975	23.49095	28.06659	33.97336	37.83182						
D61	1.380167	4.035848	11.99391	13.699	17.53849	13.43072						
D28	0.413278	0.514204	3.603404	3.728115	4.341368	5.008521						
U94	0.221982	1.599895	8.333159	7.73556	4.522964							
D32	1.575371	3.507692	5.129846	4.854435	4.354122	1.223865						
D36	1.223865	3.236725	10.5529	13.66078	16.39008	10.61988						
D93	0.431042	1.393065	2.759499	1.938168	2.074542	1.899193						

U93	0.378613	0.265617	0.269287	0.265163	0.469461	0.234903
D87	3.470383	5.898457	6.532941	4.89156	6.953566	8.573921
U87	0.508855	4.4262	1.069028	2.533226	3.684956	11.63637
C75	1.30574	0.85262	0.67639	1.163913	0.657287	0.424571
U75	1.14537	0.966531	1.593702	1.795307	1.200891	0.996441
O20	12.02047	30.23826	24.32419			
O28	4.713009	18.89861	18.38595	5.343957	3.177194	
C94	2.131856	1.441007	2.383634	3.570587		
LL6						
DMSO	1.085814	1.91664	2.079375	3.927835	3.569052	1.05464
LL8	7.580783	3.919499	2.288471	1.802625	1.78288	2.567781
AA20	9.741539	25.79155	49.99779	35.07	19.7298	29.80328
AA28	12.25819	21.71281	45.51628	21.78183	19.18808	0.728069
E94	2.628722	3.159991	1.967481	2.126174		
D86	1.921027	1.570262	1.432655	3.219622	2.415026	8.751029
F86	0.17035	0.215518	0.282999	0.337696	0.484268	0.711768
AA36	4.711737	21.29782	1.774264	10.77378		5.006065
AA24	40.22306	0.044737	0.017176	0.009595	0.044026	0.013425
AA94	3.146781	0.013148				
AA24	37.20033	50.33195	31.79067	7.632073	4.029482	0.311914
O24	21.68262	21.08162	18.4584	6.134765	0.401993	0.014254
AA60	3.475337	14.91753	0.695475			
AA61	0.656416	0.329589	0.543264	0.257434	0.577986	0.307582
C32	38.17463	90.62006	51.86994	16.43407	2.01284	2.619288
JJ28	60.46546	64.69601	71.50927	47.49171	25.81403	3.936471
C28	0.310004	0.760135	55.91046	60.63145	57.30086	6.495379
AA28	50.34727	55.18143				
U28	0.122271	44.91463	51.17075	34.01612	32.39362	3.488256
AA24*	37.20033	50.33195	31.79067	7.632073	4.029482	0.311914
AA20*	9.741539	25.79155	49.99779	35.07	19.7298	29.80328
JJ20	0.085338	40.19751	47.297	5.655104	0.142257	0.099295

AA28*		12.25819	21.71281	45.51628	21.78183	19.18808	0.728069					
JJ32		38.08705	43.77517	29.59326	0.70071	0.178921	0.70361					
D94*		14.53736	22.28715	29.68042	38.12112	42.28773	33.35092					
AA24		40.22306	0.044737	0.017176	0.009595	0.044026	0.013425					
U32		17.28347	38.37457	15.23297	0.673546	0.121004	0.15261					
D60*		7.304436	11.02975	23.49095	28.06659	33.97336	37.83182					
D24*		0.526541	2.021508	0.324894	36.64054	0.244347	0.365902					
E20		18.94919	36.04742	31.97179	24.49526	21.63777	10.55303					
O20		12.02047	30.23826	24.32419								
F32		23.8042	23.7095	14.74619	8.361793	10.90572	1.644695					
F28	0.464885	6.619239	13.95859	8.409421	7.851029	21.77709	8.655579	12.729	1.396803	0.281853	0.133842	0.149513
O24*		21.68262	21.08162	18.4584	6.134765	0.401993	0.014254					
AA36*		4.711737	21.29782	1.774264	10.77378		5.006065					
C36	7.150343	1.180274	21.27867	9.710764	5.003849	5.813189	0.164373	0.129504	0.332497	0.123413	0.062905	0.383965
E28			8.572344	20.89276		14.67007						
U94		13.0485	19.93922	20.65427								
O28		4.713009	18.89861	18.38595	5.343957	3.177194						
D61*		1.380167	4.035848	11.99391	13.699	17.53849	13.43072					
D36*		1.223865	3.236725	10.5529	13.66078	16.39008	10.61988					
AA60*		3.475337	14.91753	0.695475		6.364419	0.469952					
D70*		8.351581	5.601802	9.168348	14.68248		12.69543					
D61	0.329886	0.710035	3.970054	7.147033	10.85674	11.69027	0.787162	5.9829	7.008946	5.576734	1.343239	0.18571
JJ94		1.906203	3.87783	11.99391	0.422621	0.590515	11.63637					
U87*		0.508655	4.4262	1.069028	2.533226	3.684956	5.399266					
D60	0.604984	0.679718	3.908348	3.112999	7.65786	6.759895		1.216181	5.951785	10.45568	7.193687	0.485295
C94*		0.32801	0.908991	3.536115	10.31612		9.892867					
F94*		0.490553	4.108079	3.530146	8.596197	8.200005	9.776965					
U80*		0.119897	0.514079	2.658899	3.552453	4.456214	8.484492					
E86*		0.27629	0.248666	3.633516	6.288459	9.202082	8.751029					
D86*		1.921027	1.570262	1.432655	3.219622	2.415026						

						0.580405	2.580579	2.016952	7.131925	2.21044	0.743141
D87*	3.470383	5.898457	6.532941	4.89156	6.953566	8.573921					
U94*	0.221982	1.599895	8.333159	7.73556	4.522964	2.567781					
LL8	7.580783	3.919499	2.288471	1.802625	1.78288						
E24	0.098517	0.057019	7.404745	1.12791	0.329197	0.326384					
M17	0.433646	0.484853	0.515332	0.672973	0.763891	1.360645					
LL6	0.809416	0.854576	1.812577	2.932931	3.939451	6.879564					
D25*	0.105104	0.352229	3.212175	6.167455	1.617246	1.792186					
E32	5.170892	6.057429	4.380769	1.365759	0.845009	0.111133					
D32*	1.575371	3.507692	5.129846	4.854435	4.354122	1.223865					
LL8*	1.366191	1.875091	4.594308	4.423822	4.416413	5.083137					
JJ36	2.339652	5.039758	2.000554	0.284902	0.159879	0.033605					
D28*	0.413278	0.514204	3.603404	3.728115	4.341368	5.008521					
U36	0.968904	2.501669	4.650123	2.269889	1.310025	0.23654					
E80	1.697744	2.624376	3.868368	3.757709	4.412999	1.895016					
E36	1.044698	2.940734	4.27308	1.367514	1.194649	0.11883					
LL6*	1.085814	1.91664	2.079375	3.927835	3.569052	1.05464					
C94*	2.131856	1.441007	2.383634	3.570587	3.173297						
JJ80	0.5771	0.4786	2.199535	3.226941		2.653163					
E94*	2.628722	3.159991	1.967481	2.126174							
AA94*	3.146781	0.013148									
D93*	0.431042	1.393065	2.759499	1.938168	2.074542	1.899193					
B17	0.72135	0.44715	0.996508	1.067002	1.098275	2.197846					
C86	0.159044	0.279389	0.637804	1.055779	1.025192	1.422756					
U75*	1.14537	0.966531	1.593702	1.795307	1.200891	0.996441					
JJ86	0.503544	0.775429	0.794641	0.537706	1.491694	0.707425					
C86	0.159044	0.279389	0.637804	1.055779	1.025192	1.422756					
C75*	1.30574	0.85262	0.67639	1.163913	0.657287	0.424571					
C20	0.126573	0.098883	0.088576	1.003372	0.11883	0.138726					
C80*	0.578045	0.102763	0.111304	0.528832	0.887811	0.961981					
F86*	0.17035	0.215518	0.282999	0.337696	0.484268	0.711768					

M17*	0.550188	0.409299	0.304933	0.35066	0.404506	0.356775	0.392121	0.376042	0.34777	0.315241	0.468629	0.705352
AA61*		0.656416	0.329589	0.543264	0.257434	0.577986	0.307582					
B17*		0.614141	0.505597	0.339801	0.319609	0.399828	0.406147					
U28		'	0.221192	0.515945	0.290904	0.237264						
U93*		0.378613	0.265617	0.269287	0.265163	0.469461	0.234903					
U86*		0.435638	0.196901	0.124174	0.304979	0.193936	0.189116					
II36		0.331156	0.344857	0.209068	0.107622	0.151587	0.170145					
II28		0.334806	0.137402	0.079535	0.165612	0.097702	0.126852					
JJ24		0.199154	0.188046	0.090045	0.064921	0.085969	0.032536					
C25*		0.11105	0.044342	0.06737	0.031668	0.088061	0.05876					
U25		0.055718	0.056074	0.069147	0.047859	0.04537	0.088747					
II32		0.046949	0.026455	0.070335	0.047503	0.033368	0.0261					

*Polymers with a asterisk were synthesize with DMSO (2 mL) added to the reaction mixture.
Other polymers were synthesized neat.

Claims

5   What is claimed is:

1.   A compound of the formula:
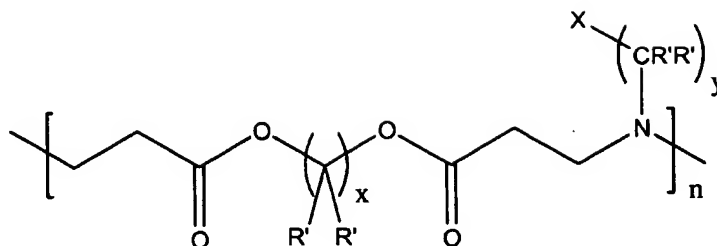
10   wherein

           X is methyl, OR or NR_2;

           R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl,

     cyclic, heterocyclic, aryl, and heteroaryl;

           each R' is independently selected from the group consisting of hydrogen, C_1-C_6

15   lower alkyl, C_1-C_6 lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano,

     thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen;

           n is an integer between 3 and 10,000;

           x is an integer between 1 and 10;

           y is an integer between 1 and 10; and

20   derivatives and salts thereof.

2.   The compound of claim 1, wherein the compound is amine-terminated.

3.   The compound of claim 1, wherein the compound is acrylate-terminated.

25

4.   The compound of claim 1, wherein x is an integer between 2 and 7.

5.   The compound of claim 1, wherein x is 4.

5   6.   The compound of claim 1, wherein x is 5

7.   The compound of claim 1, wherein x is 6.

8.   The compound of claim 1, wherein y is an integer between 2 and 7.

10

9.   The compound of claim 1, wherein y is 4.

10.   The compound of claim 1, wherein y is 5.

15 · 11.   The compound of claim 1, wherein y is 6.

12.   The compound of claim 1, wherein R is hydrogen.

13.   The compound of claim 1, wherein R is methyl, ethyl, propyl, butyl, pentyl, and
20   hexyl.

14.   The compound of claim 1, wherein the structure of the compound is

C28
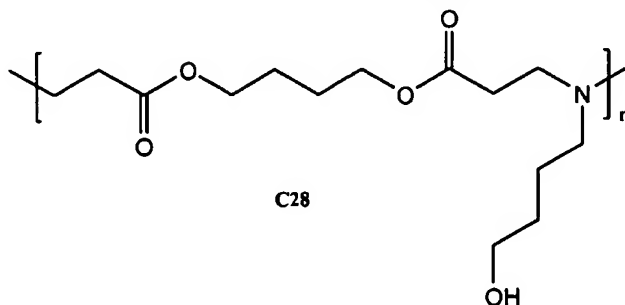
wherein R is hydrogen, x is 4, and y is 4.

25

15.   The compound of claim 1, wherein the structure of the compound is

E28
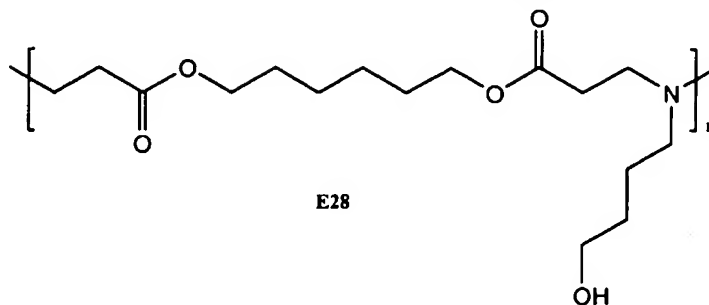
wherein R is hydrogen, x is 6, and y is 4.

16.   The compound of claim 1, wherein the structure of the compound is

C32
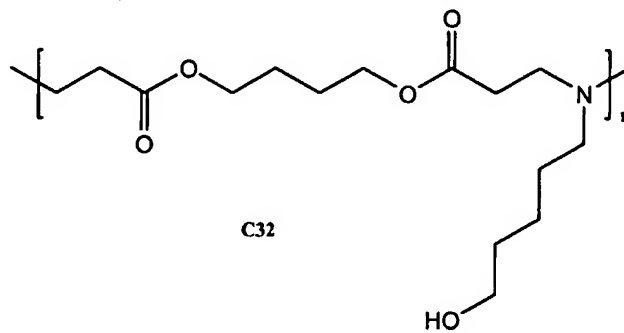
wherein R is hydrogen, x is 4, and y is 5.

17.   The compound of claim 1, wherein the structure of the compound is

JJ28
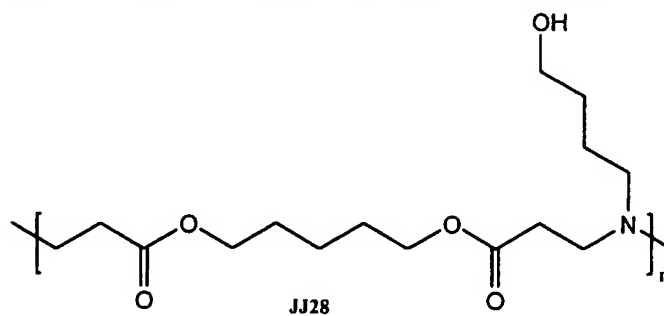
wherein R is hydrogen, x is 5, and y is 4.

18. The compound of claim 1, wherein the structure of the compound is

JJ32
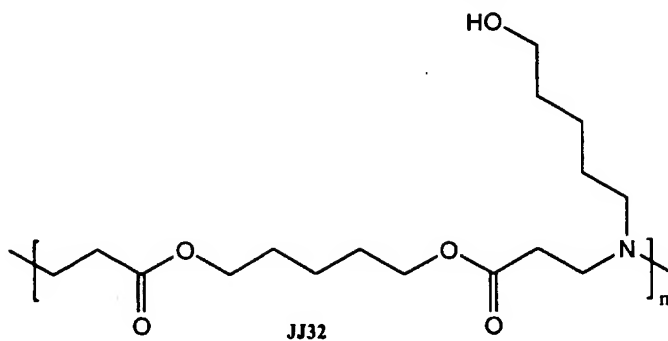
5   wherein R is hydrogen, x is 5, and y is 5.

19. The compound of claim 1, wherein the structure of the compound is

JJ36
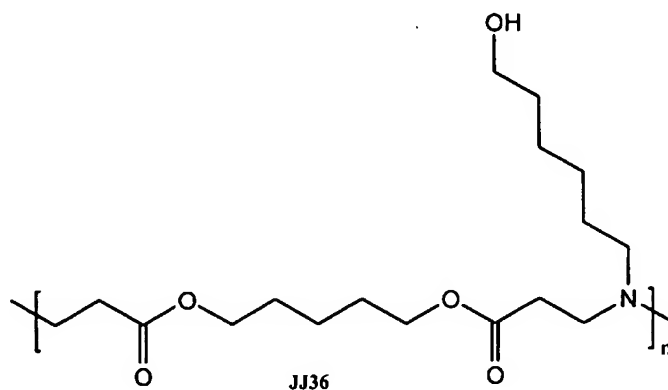
wherein R is hydrogen, x is 5, and y is 6.

10

20. A compound of the formula:

D94
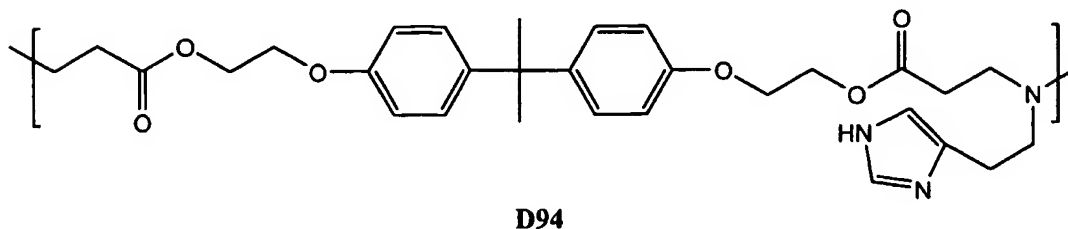
wherein n is an integer between 3 and 10,000; and derivatives and salts thereof.

15

21.   A compound of the formula:

KK89
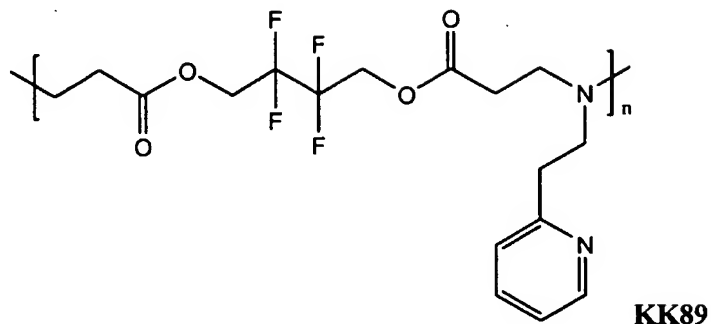
wherein n is an integer between 3 and 10,000; and derivatives and salts thereof.

22.   A compound of the formula:

M17
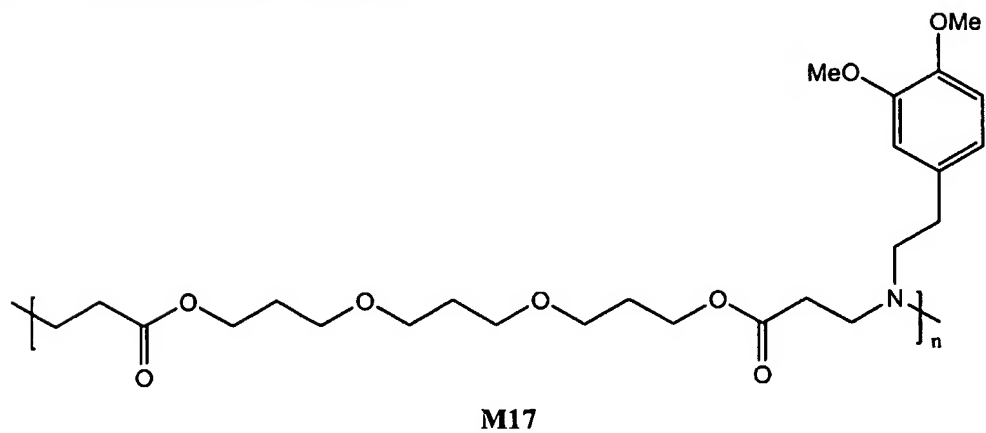
wherein n is an integer between 3 and 10,000; and derivatives and salts thereof.

23.   A compound of the formula:

C36
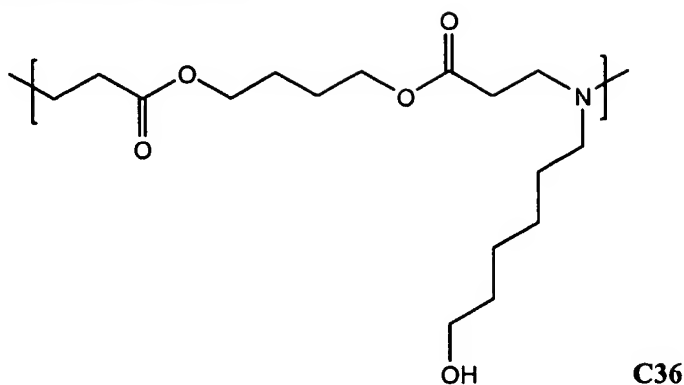
5

wherein n is an integer between 3 and 10,1000; and derivatives and salts thereof.

24.   A polymer selected from the group consisting of:

10   C86, D60, B14, G5, D61, U94, F32, F28, JJ36, JJ32, LL6, LL8, U28, E28, U36,
E36, U32, E32, C94, F94, JJ94, U28, JJ86, C86, U86, E86, C80, E80, JJ80, U80,
D24, E24, JJ24, B17, II28, II36, II32, C20, JJ20, E20, C25, U25, D25, D70, D28,
D32, D36, D93, U87, D87, C75, U75, O20, O28, C94, AA20, AA28, D86, F86,
AA36, AA24, AA94, O24, AA60, A61, C32, JJ28, C28, JJ20, D94, U32, D24, C36,

15   E28, D36, U94, E24, E32, D28, U36, E80, E36, JJ80, E94, D93, B17, M17, AA61,
U93, and C25;

wherein the acrylates A-PP are of the formula:

A, B, C, D, E, F, J, K, L, M, O

P, Q, R, S, T, U, Z

AA, BB, II, JJ, KK, LL, PP

;
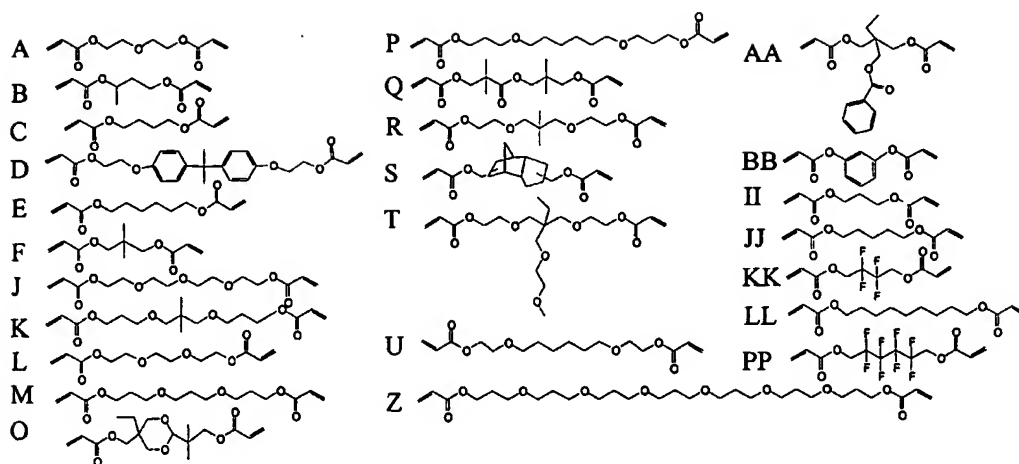
and

the amines **1-94** are of the formula:

1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18

19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39

40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59

60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77

78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94

5
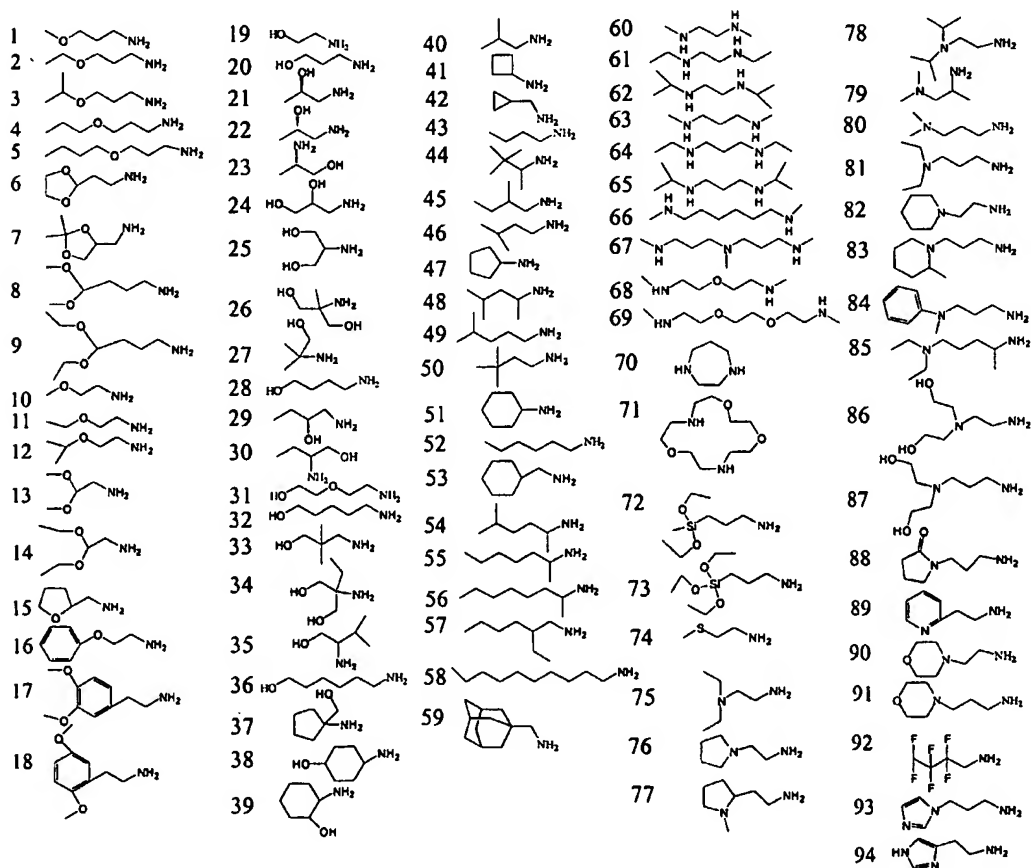
25.   A poly(beta-amino ester) comprising a bis(acrylate ester) selected from the group consisting of formulae **A-PP**:
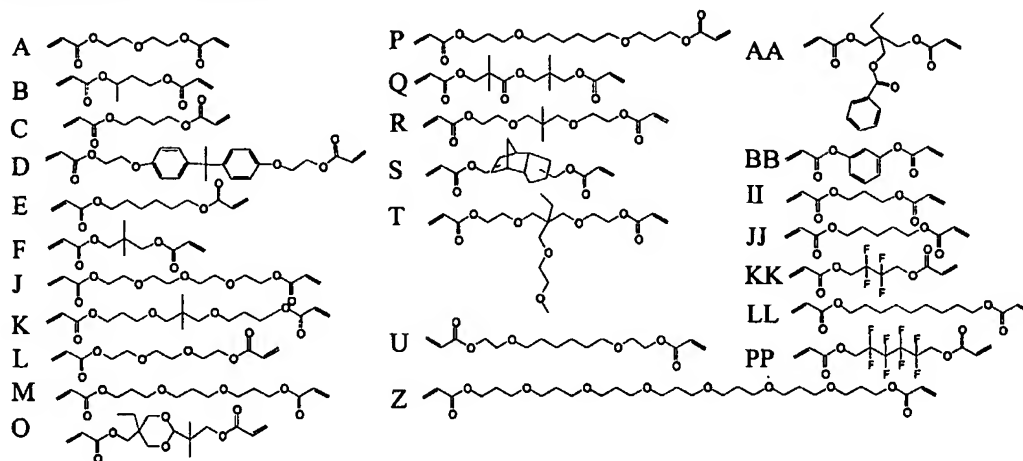
5   26.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of the formula:

B.
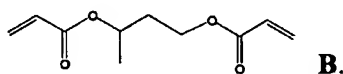
27.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of the

10   formula:

C.
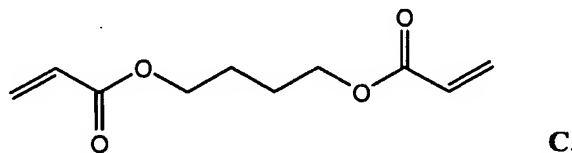
28.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of the formula:

15

D.
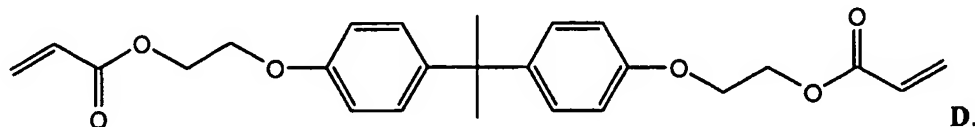
29.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of the

formula:

F.
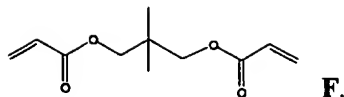
30.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of

formula:

M.
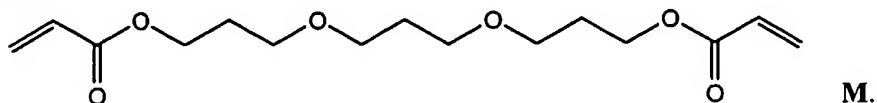
31.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of

formula:

U.
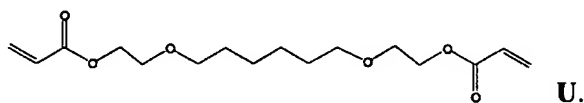
32.   A poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of

formula:

E.
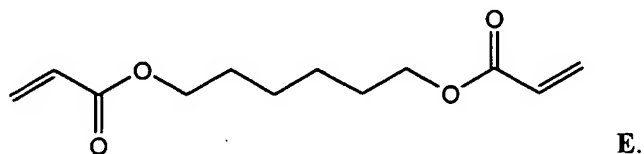
33.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of

formula:

AA.
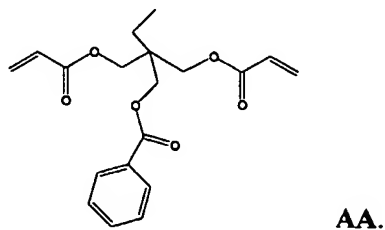
34.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of formula:

JJ.
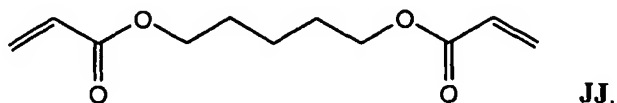
35.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of formula:

KK.
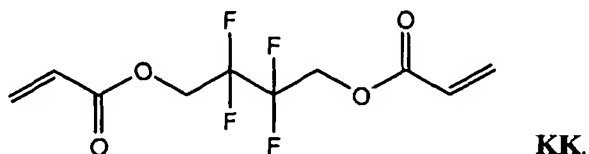
36.   The poly(beta-amino ester) of claim 25 comprising a bis(acrylate ester) of formula:

LL.
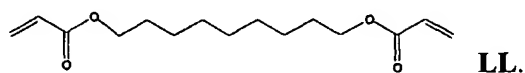
37.   A poly(beta-amino ester) comprising an amine selected from the group consisting of the formulae 1-94:

Numbered amine structures 1–94.
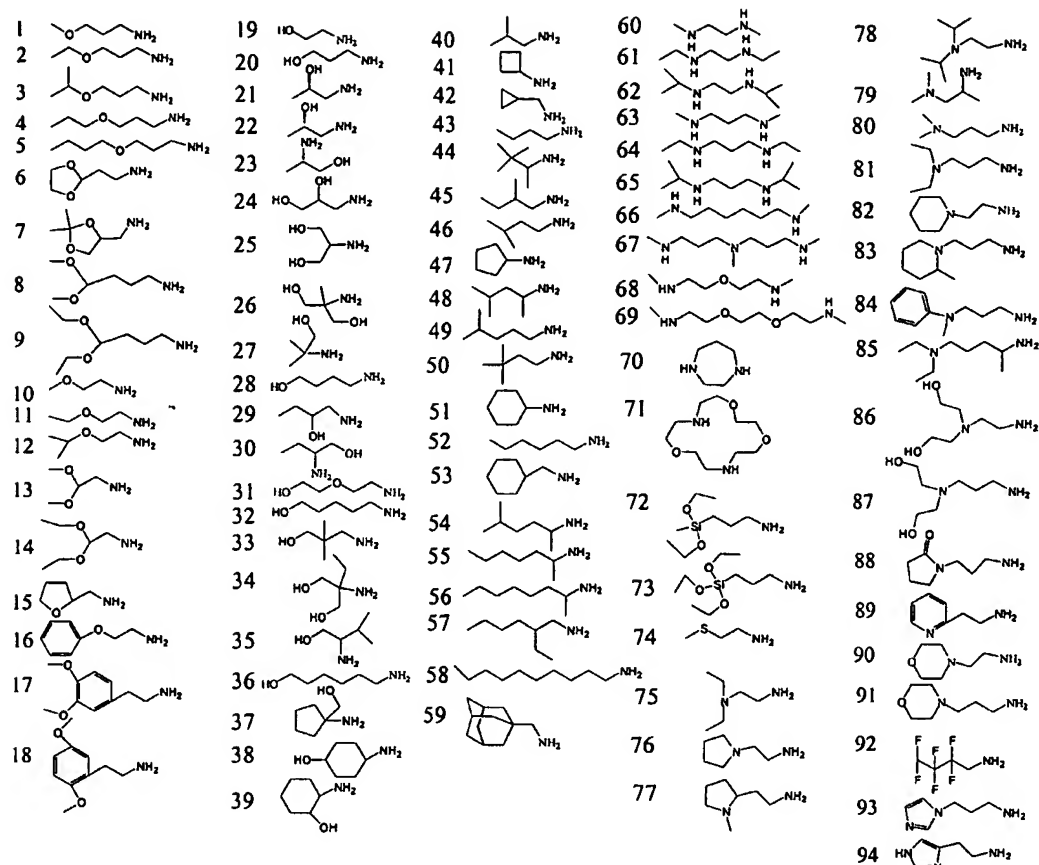
38.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

6.
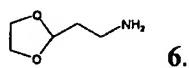
39.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

8.
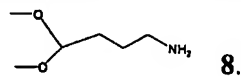
40.   The poly(beta-amino ester) of claim 37 comprising an amine of the formula:

17.
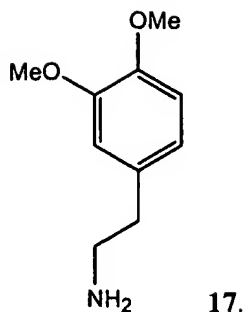
41.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

HO⌒⌒NH₂ **20.**
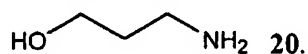
42.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

24.
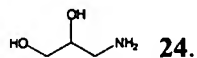
43.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:
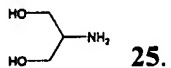
25.

44.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

HO⌒⌒⌒NH₂ **28.**
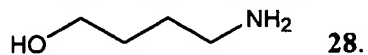
45.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

HO⌒⌒⌒⌒NH₂ **32.**
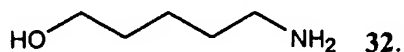
46.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

HO⌒⌒⌒⌒⌒NH₂ **36.**
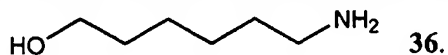
47.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

60.
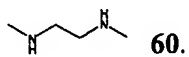
48.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

 61.
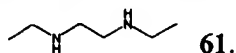
5   49.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

 70.
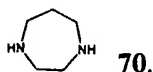
50.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

 75.
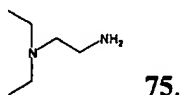
10

51.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

 80.
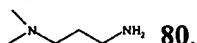
52.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

15

 86.
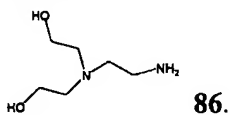
53.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

 87.
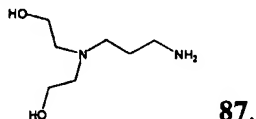
20

54.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

 89.
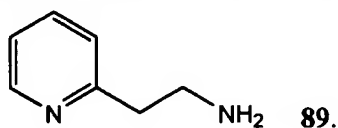
55.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

93.
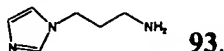
56.   The poly(beta-amino ester) of claim 37 comprising an amine of formula:

94.

5
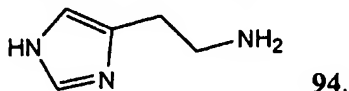
57.   The compound of claim 1, wherein the compound has a molecular weight between 1,000 and 100,000 g/mol.

10   58.   The compound of claim 1, wherein the compound has a molecular weight between 2,000 and 40,000 g/mol.

59.   A pharmaceutical composition comprising a polynucleotide and a compound of claim 1.

15

60.   A pharmaceutical composition comprising nanoparticles containing a polynucleotide and a compound of claim 1.

61.   A pharmaceutical composition comprising nanoparticles containing a

20   pharmaceutical agent and a compound of claim 1.

62.   A pharmaceutical composition comprising microparticles containing an agent encapsulated in a matrix of a compound of claim 1.

25   63.   The pharmaceutical composition of claim 62 wherein the microparticles have a mean diameter of 1-10 micrometers.

64.   The pharmaceutical composition of claim 62 wherein the microparticles have a mean diameter of less than 5 micrometers.

65.   The pharmaceutical composition of claim 62 wherein the microparticles have a mean diameter of less than 1 micrometer.

5   66.   The pharmaceutical composition of claim 62 wherein the agent is a polynucleotide.

67.   The pharmaceutical composition of claim 62 wherein the polynucleotide is DNA.

10

68.   The pharmaceutical composition of claim 62 wherein the polynucleotide is RNA.

69.   The pharmaceutical composition of claim 62 wherein the polynucleotide is an

15   siRNA.

70.   The pharmaceutical composition of claim 62 wherein the agent is a small molecule.

20   71.   The pharmaceutical composition of claim 62 wherein the agent is a peptide.

72.   The pharmaceutical composition of claim 62 wherein the agent is a protein.

25   73.   A composition comprising
                a poly(beta-amino ester),
                a polynucleotide, and
                a co-complexing agent selected from the group consisting of cationic
        polymers, cationic proteins, PLGA, spermine, spermidine, polyamines,
30   polyethyleneimine (PEI), and polylysine(PLL).

74.   The composition of claim 73, wherein the ratio of co-complexing agent to polynucleotide ranges from 0.1 to 1.2 (w/w/w).

75.   The composition of claim 73, wherein the ratio of poly(beta-amino ester) to
5   polynucleotide ranges from 10 to 120.

76.   A method of synthesizing a poly(β-amino ester), the method comprising steps of:

    providing a primary amine or bis(secondary amine);

10   providing a bis(acrylate ester); and

    reacting the amine and the bis(acrylate ester) under suitable conditions to form the poly(β-amino ester).

77.   The method of claim 76, further comprising the step of screening the polymer
15   for a desired characteristic, wherein the poly(β-amino ester) is not precipitated , purified, or isolated before the step of screening.

78.   The method of claim 76, wherein the step of reacting comprises reacting the amine and the bis(acrylate ester) in an organic solvent.

20

79.   The method of claim 76, wherein the step of reacting comprises reacting the amine and the bis(acrylate ester) in the absence of a solvent.

80.   The method of claim 76, wherein the organic solvent is selected from the group

5   consisting of THF, diethyl ether, glyme, hexanes, methanol, ethanol, isopropanol,

methylene chloride, chloroform, carbon tetrachloride, dimethylformamide, acetonitrile,

benzene, DMSO, and toluene.

81.   The method of claim 76, wherein the organic solvent is DMSO.

10

82.   The method of claim 76, wherein the concentration of the amine is between

approximately 0.01 M and 5 M.

83.   The method of claim 76, wherein the concentration of the amine is between

15   approximately 0.1 M and 2 M.

84.   The method of claim 76, wherein the concentration of the amine is between

approximately 1 M and 2 M.

20   85.   The method of claim 76, wherein the concentration of the bis(acrylate ester) is

between approximately 0.01 M and 5 M.

86.   The method of claim 76, wherein the concentration of the bis(acrylate ester) is

between approximately 0.1 M and 2 M.

25

87.   The method of claim 76, wherein the concentration of the bis(acrylate ester) is

between approximately 1 M and 2 M.

30   88.   The method of claim 76, wherein the step of reacting comprises reacting the

amine and the bis(acrylate ester) at a temperature between 0 and 75 °C.

89.　　The method of claim 76, wherein the step of reacting comprises reacting the amine and the bis(acrylate ester) at a temperature between 20 and 50 °C.

5　90.　　The method of claim 76, wherein the step of reacting comprises reacting the amine and the bis(acrylate ester) at a temperature between 30 and 60 °C.

91.　　A method of encapsulating an agent in a matrix of poly(β-amino esters) to form microparticles, the method comprising steps of:

10　　　　　　　providing an agent;

　　　　　　　providing a poly(β-amino ester); and

　　　　　　　contacting the agent and the poly(β-amino ester) under suitable conditions to form microparticles.

15　92.　　The method of claim 91 wherein the agent is a polynucleotide.

93.　　The method of claim 92 wherein the polynucleotide is DNA.

94.　　The method of claim 92 wherein the polynucleotide is RNA.

20

95.　　The method of claim 91 wherein the agent is a small molecule.

96.　　The method of claim 91 wherein the agent is a protein.

25　97.　　The method of claim 91 wherein the poly(β-amino ester) is a compound of claim 1.

98.　　The method of claim 91 wherein the step of contacting comprises spray drying a mixture of the agent and the poly(β-amino ester).

30

99.   The method of claim 91 wherein the step of contacting comprises double emulsion solvent evaporation techniques.

100.   The method of claim 91 wherein the step of contacting comprises a phase
5   inversion technique.

101.   A method of screening a library of polymers, the method comprising steps of:
          providing a plurality of polymers, wherein the polymers are not polynucleotides or proteins; and
10   screening the polymers for a desired property useful in gene therapy.

102.   A method of screening a library of polymers, the method comprising steps of:
          providing a plurality of poly(β-amino esters); and
          screening the polymers for a desired property.

15

103.   The method of claim 101, wherein the step of providing comprises synthesizing the polymers in parallel.

104.   The method of claim 103, wherein the step of synthesizing comprises
20   synthesizing the polymers in DMSO.

105.   The method of claim 103, wherein the step of synthesizing does not include precipitating, purifiying, or isolating the polymer before the step of screening.

25   106.   The method of claim 101, wherein the plurality of poly(beta-amino ester)s comprises at least 500 poly(beta-amino ester)s.

107.   The method of claim 101, wherein the plurality of poly(beta-amino ester)s comprises at least 1000 poly(beta-amino ester)s.

30

108.   The method of claim 101, wherein the plurality of poly(beta-amino ester)s

comprises at least 1500 poly(beta-amino ester)s.

109.   The method of claim 101, wherein the plurality of poly(beta-amino ester)s comprises at least 2000 poly(beta-amino ester)s.

5

110.   The method of claim 101, wherein the desired property is an ability to bind a polynucleotide.

111.   The method of claim 101, wherein the desired property is solubility in an
10   aqueous solution.

112.   The method of claim 101, wherein the desired property is solubility in an aqueous solution at a pH lower than 7.

15   113.   The method of claim 101, wherein the desired property is solubility in an aqueous solution at pH 5 and not being soluble in an aqueous solution at pH 7.

114.   The method of claim 101, wherein the desired property is an ability to bind heparin.
20

115.   The method of claim 101, wherein the desired property is an ability to increase transfection efficiency.

116.   The method of claim 101, wherein the desired property is useful in tissue
25   engineering.

117.   The method of claim 118, wherein the desired property is the ability to support cell growth.

30   118.   The method of claim 101, wherein the desired property is the ability to support cell attachment.

119.   The method of claim 101, wherein the desired property is the ability to support tissue growth.

5

120.   A method of screening a collection of polymers, the method comprising steps of:

    providing a plurality of polymers;

    providing a polynucleotide including a reporter gene;

10   providing cells;

    contacting each of the polymers with the polynucleotide resulting in a polynucleotide:polymer mixture;

    contacting the cells with polynucleotide:polymer mixture; and

    determining whether cells are expressing the reporter gene.

15

121.   The method of claim 120, wherein the step of providing a plurality of polymers comprises synthesizing the polymers in parallel.

122.   The method of claim 120, wherein the polymers are poly(beta-amino esters).

20

123.   The method of claim 120, wherein the polymers are polyesters.

124.   The method of claim 120, wherein the polymers are polyamides.

25   125.   The method of claim 120, wherein the plurality of polymers comprises at least 500 different polymers.

126.   The method of claim 120, wherein the plurality of polymers comprises at least

30   1000 different polymers.

127. The method of claim 120, wherein the reporter gene encodes green fluorescent protein.

128. The method of claim 120, wherein the cells are mammalian cells.

5

129. The method of claim 120, wherein the cells are bacterial cells.

130. The method of claim 120, wherein the cells are COS-7 cells.

10   131. The method of claim 120, wherein the ratio of polymer to polynucleotide is 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1, 95:1, 100:1, 105:1, 110:1, 120:1, 130:1, 140:1, 150:1, and 200:1.

132. The method of claim 120, wherein the step of determining comprises
15   quantifying the amount of reporter gene expression.
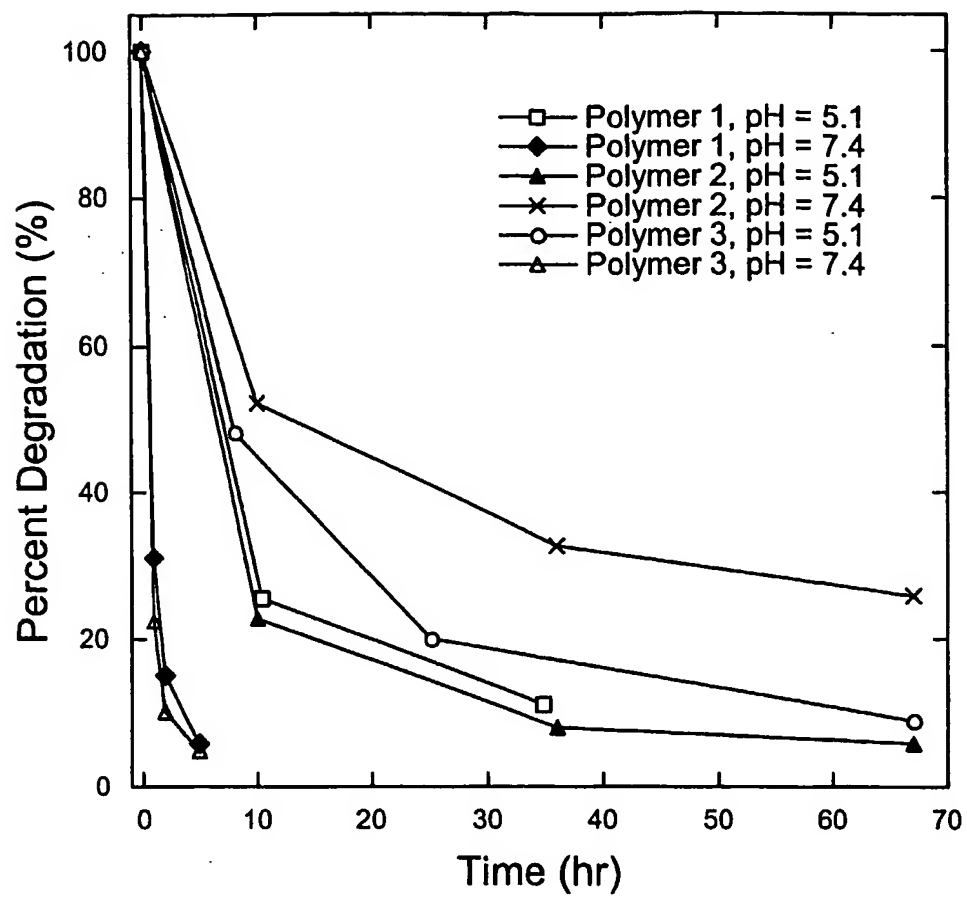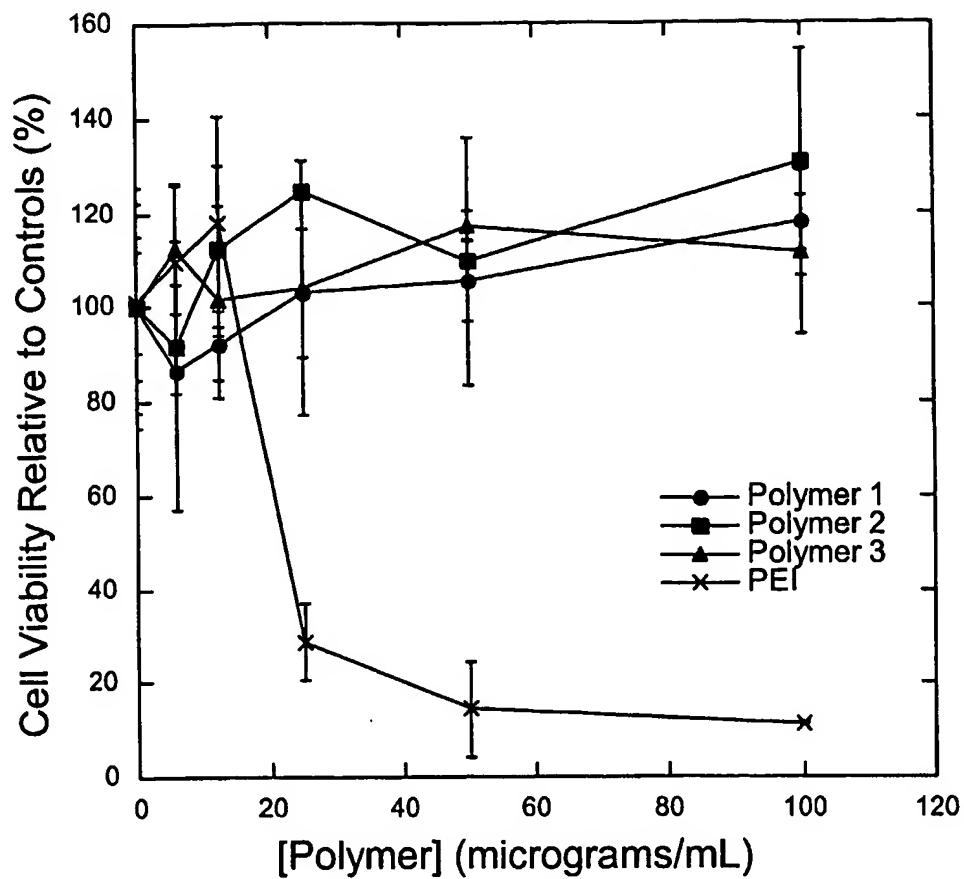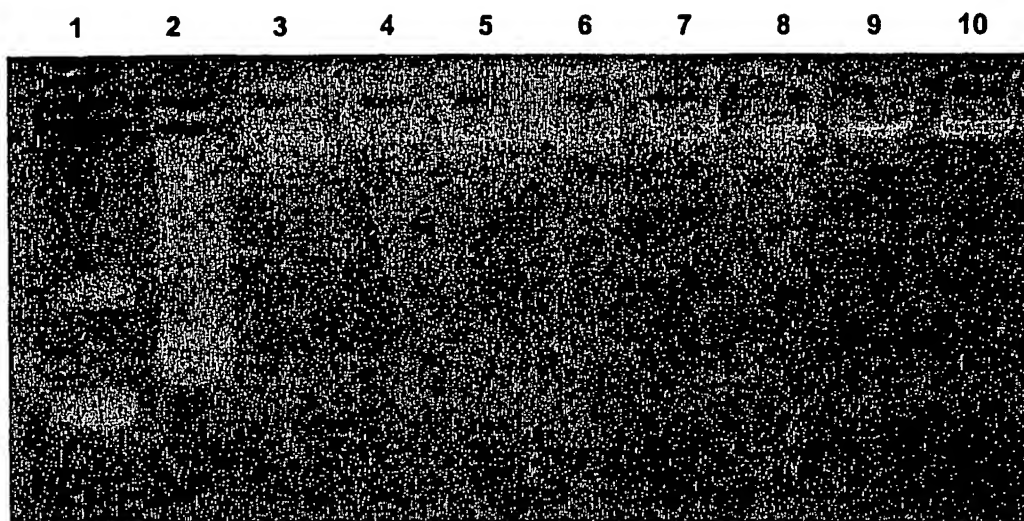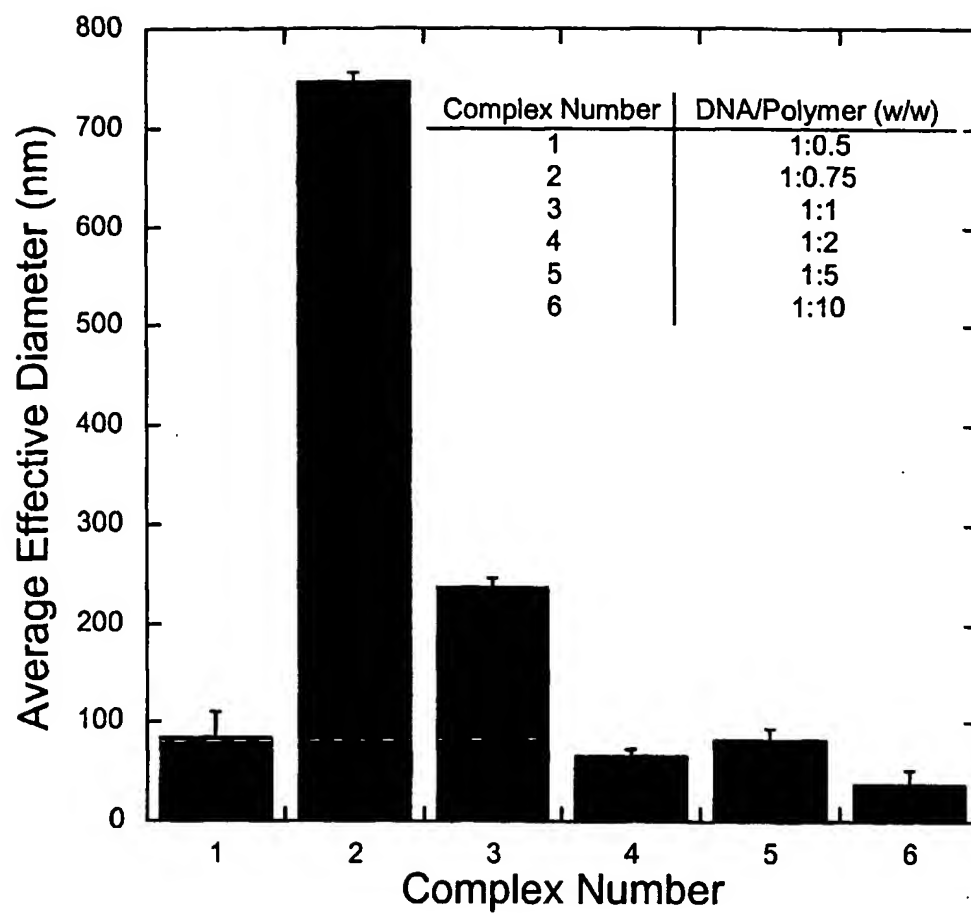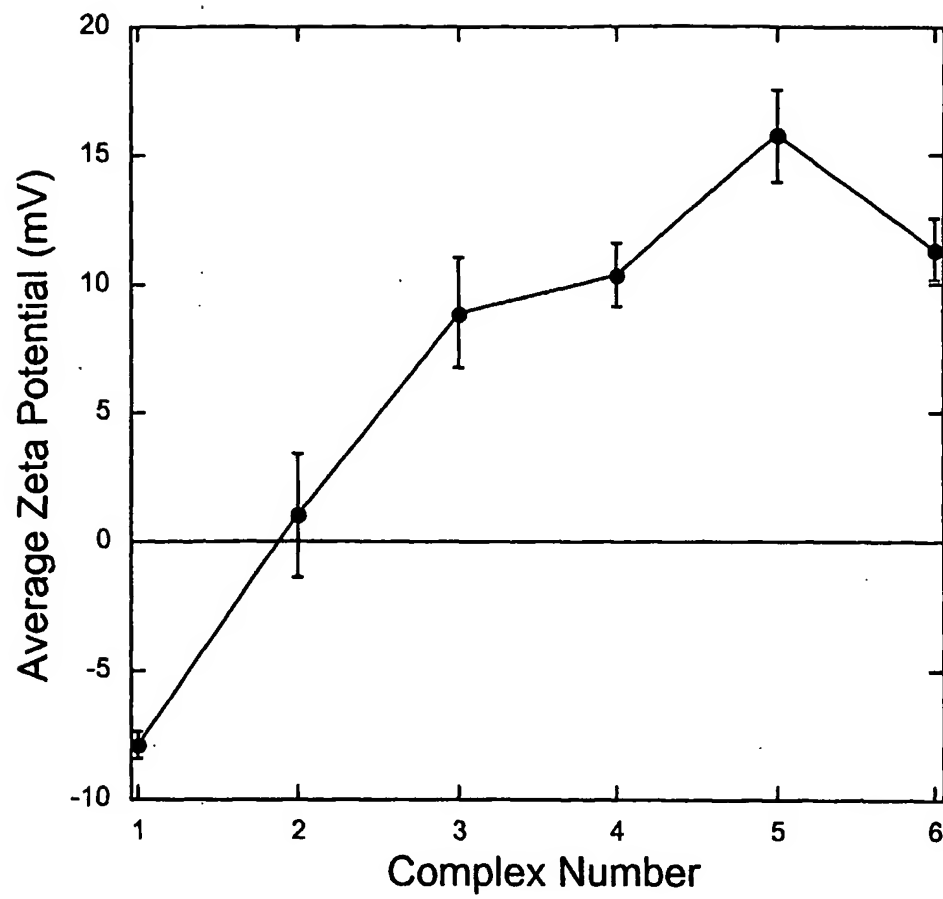
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

poly-**1**
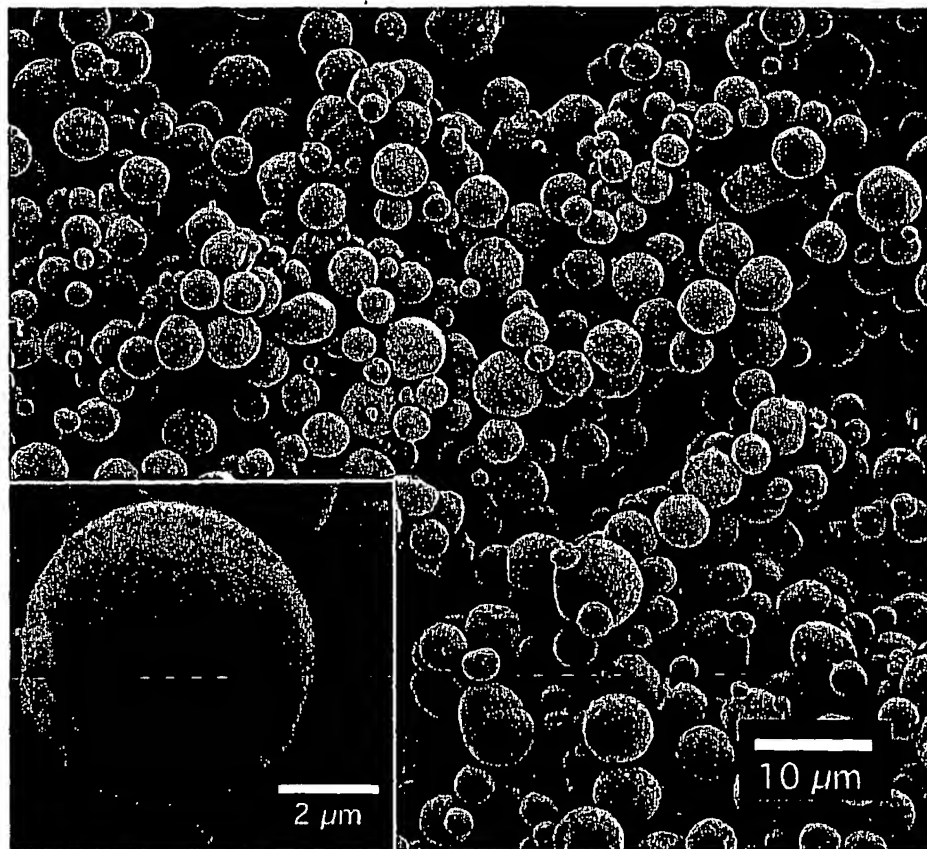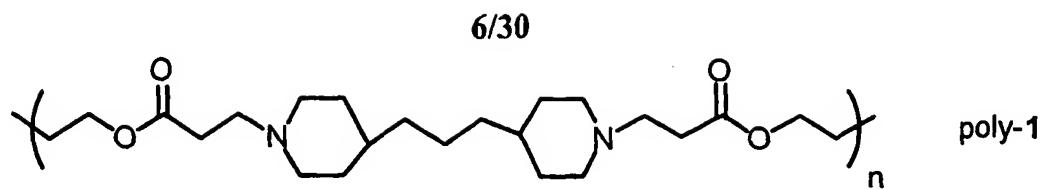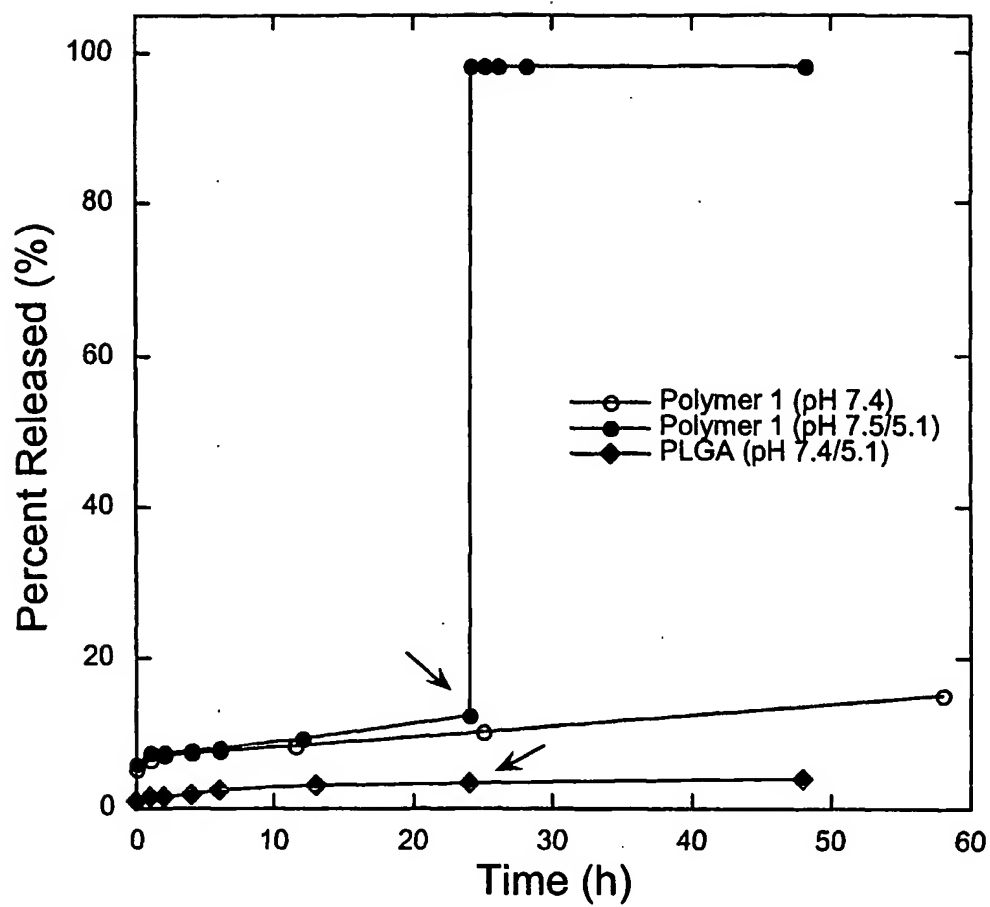
Figure 6

Figure 7

FIG. 8A
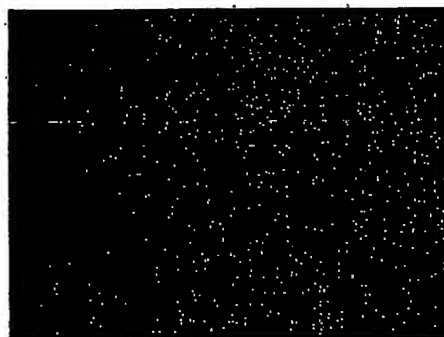
FIG. 8B
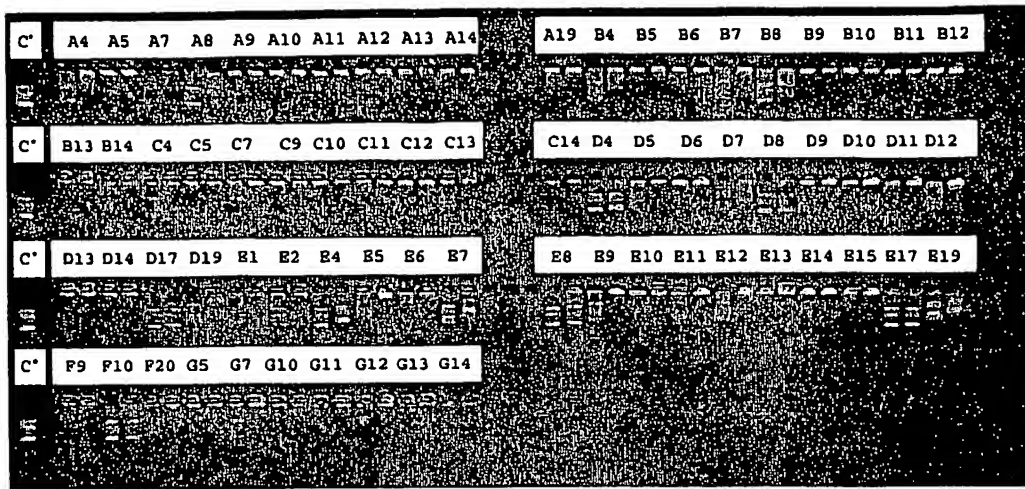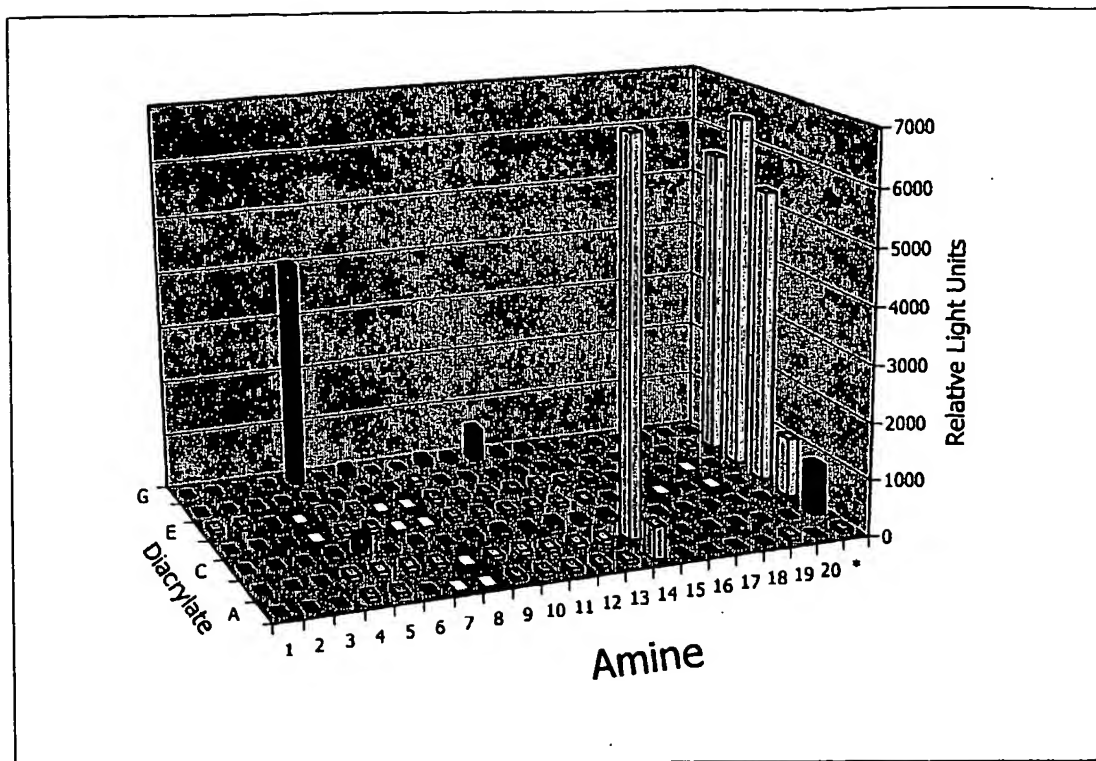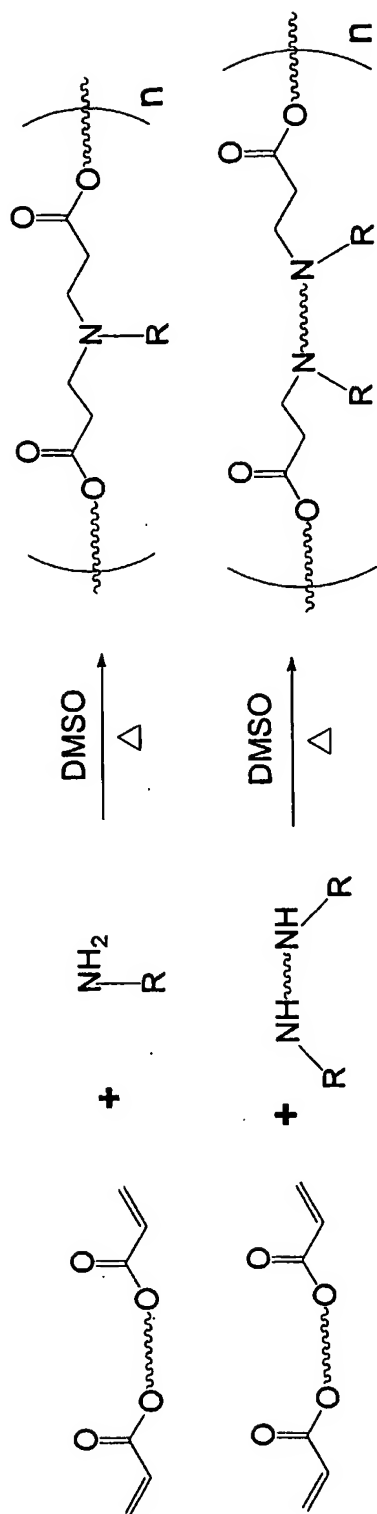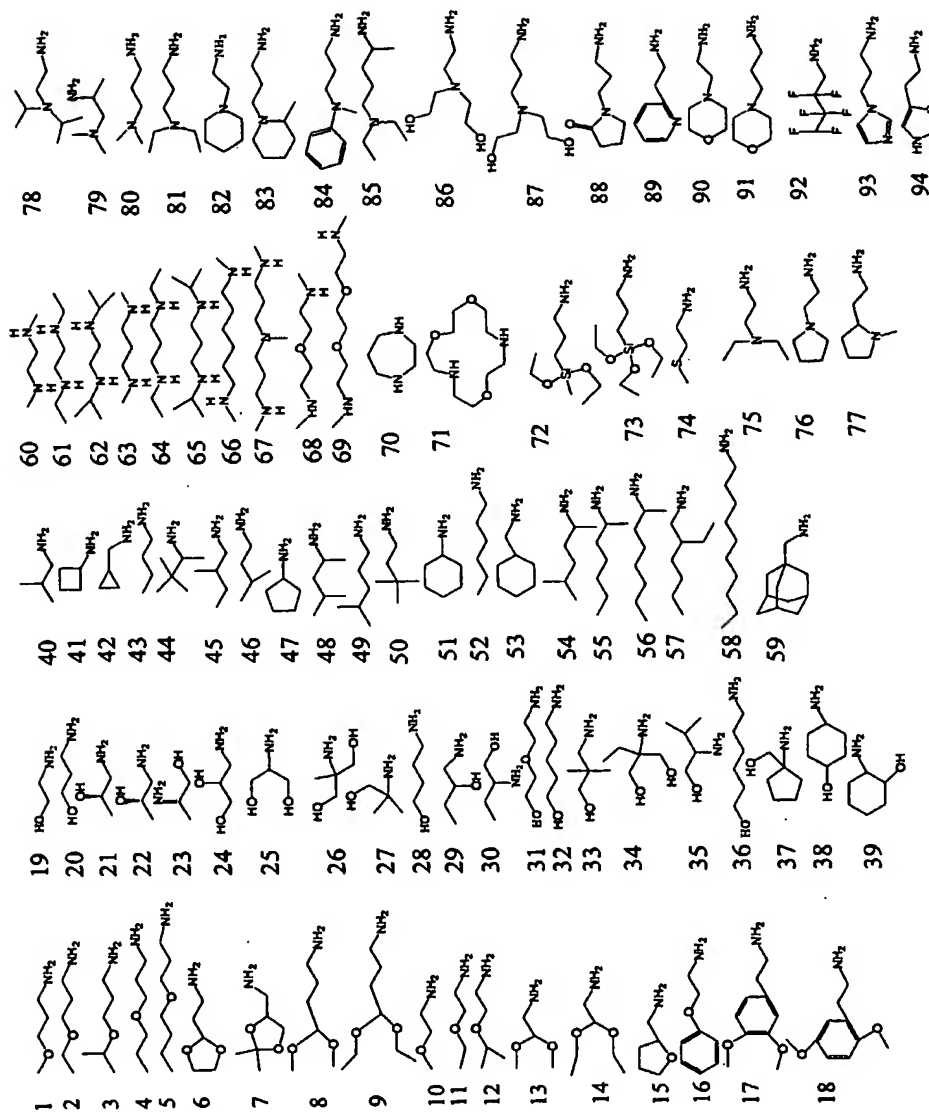
Figure 9
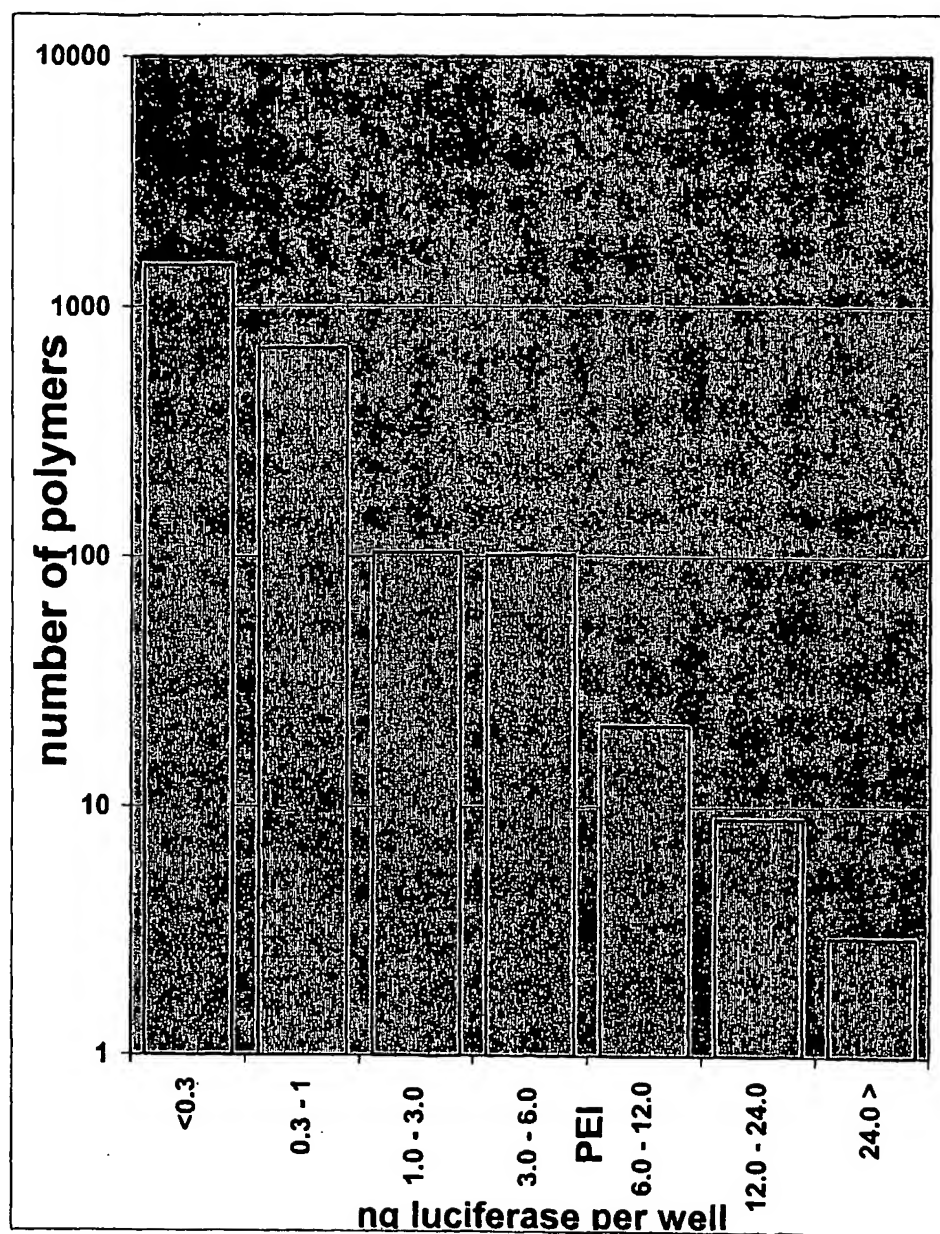
Figure 10

Figure 11
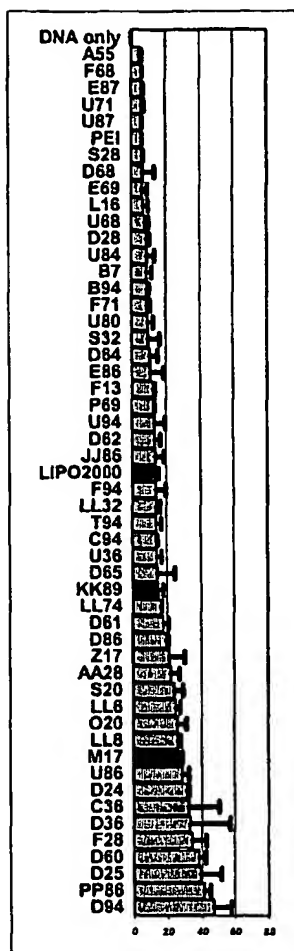
Figure 12

Figure 13

Figure 14

Polymer	Binding
A55	+
F68	+
E87	+
U71	+
U87	+
S28	+
D68	+
E69	+
L16	(+/-)
U68	+
D28	+
U84	+
B7	(+/-)
F71	+
U80	+
S32	+
D64	+
E86	+
F13	(+/-)
P69	+
U94	+
D62	+
JJ86	+
F94	+
LL32	+
T94	+
C94	+
U36	+
D65	+
KK89	-
LL74	+
D61	+
D86	+
Z17	+
AA28	+
S20	+
LL6	+
O20	+
LL8	+
M17	-
U86	+
D24	+
C36	+
D36	+
F28	+
D60	+
D25	+
PP86	+
D94	+

Figure 15

Figure 16

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B

Figure 17

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B
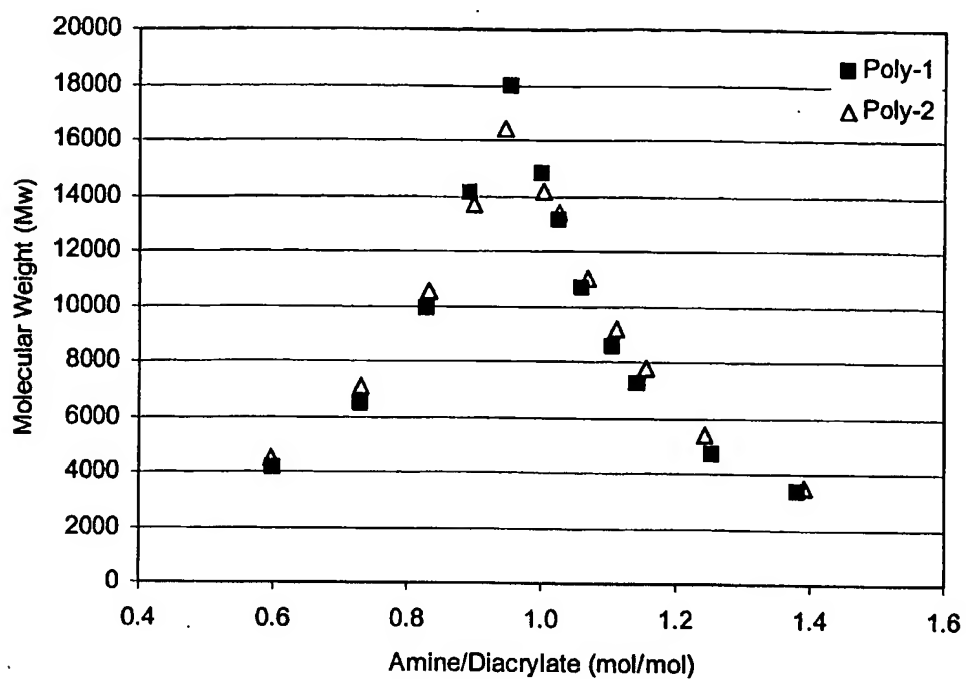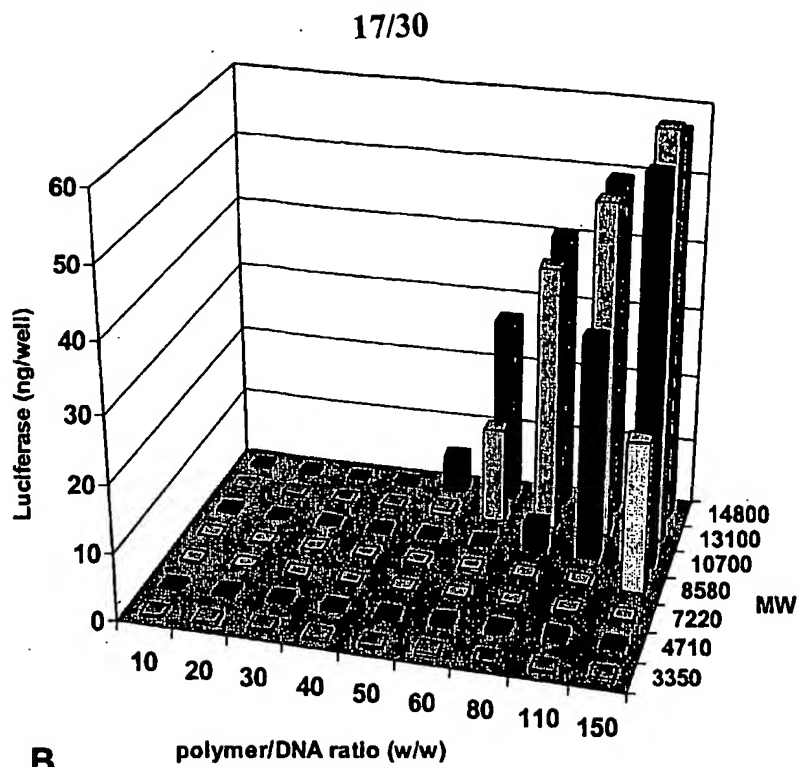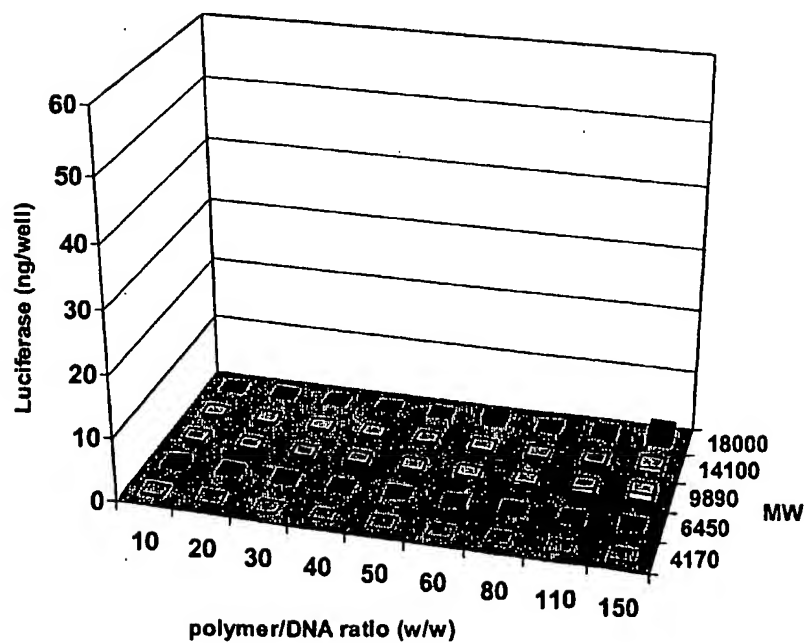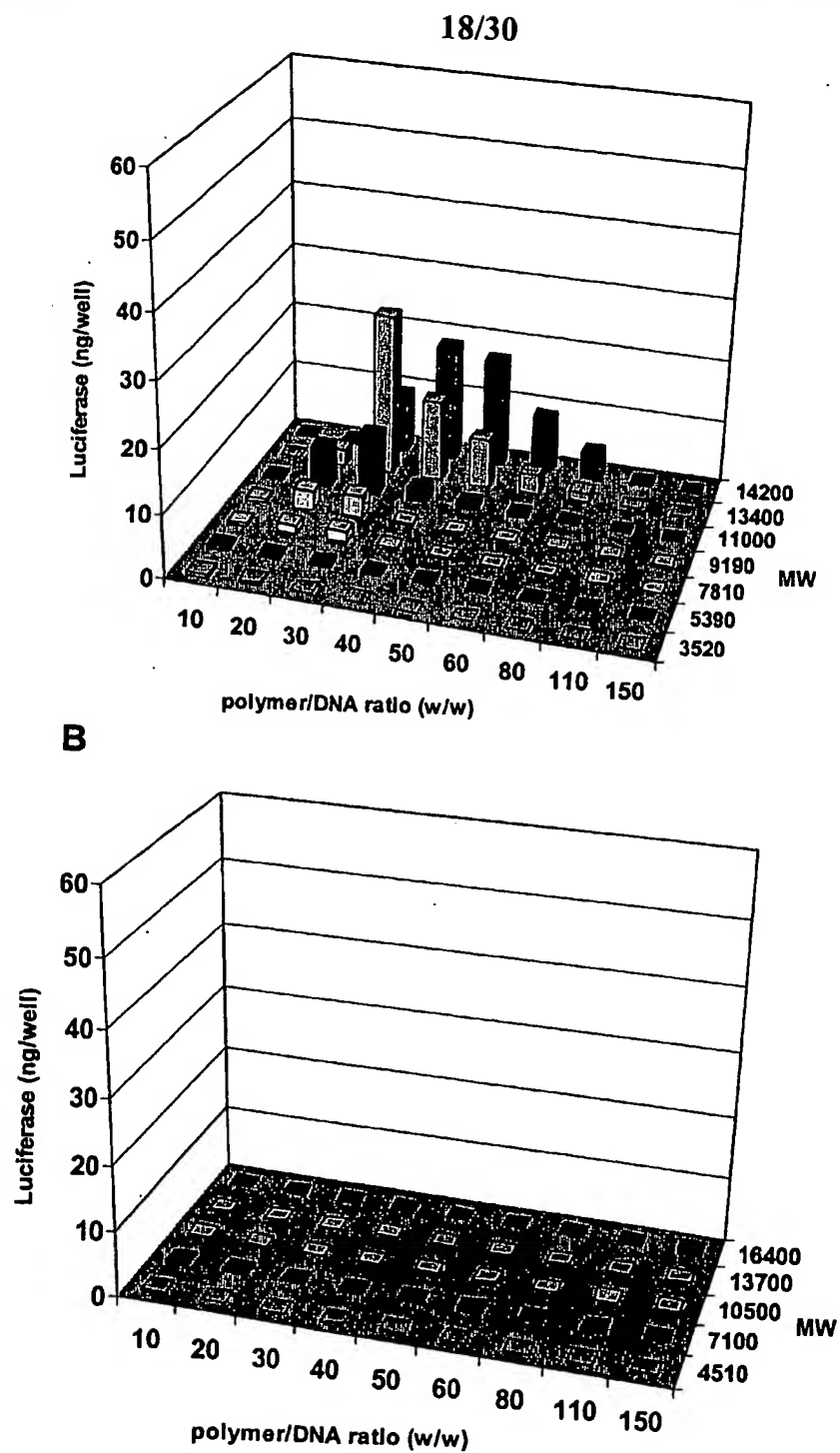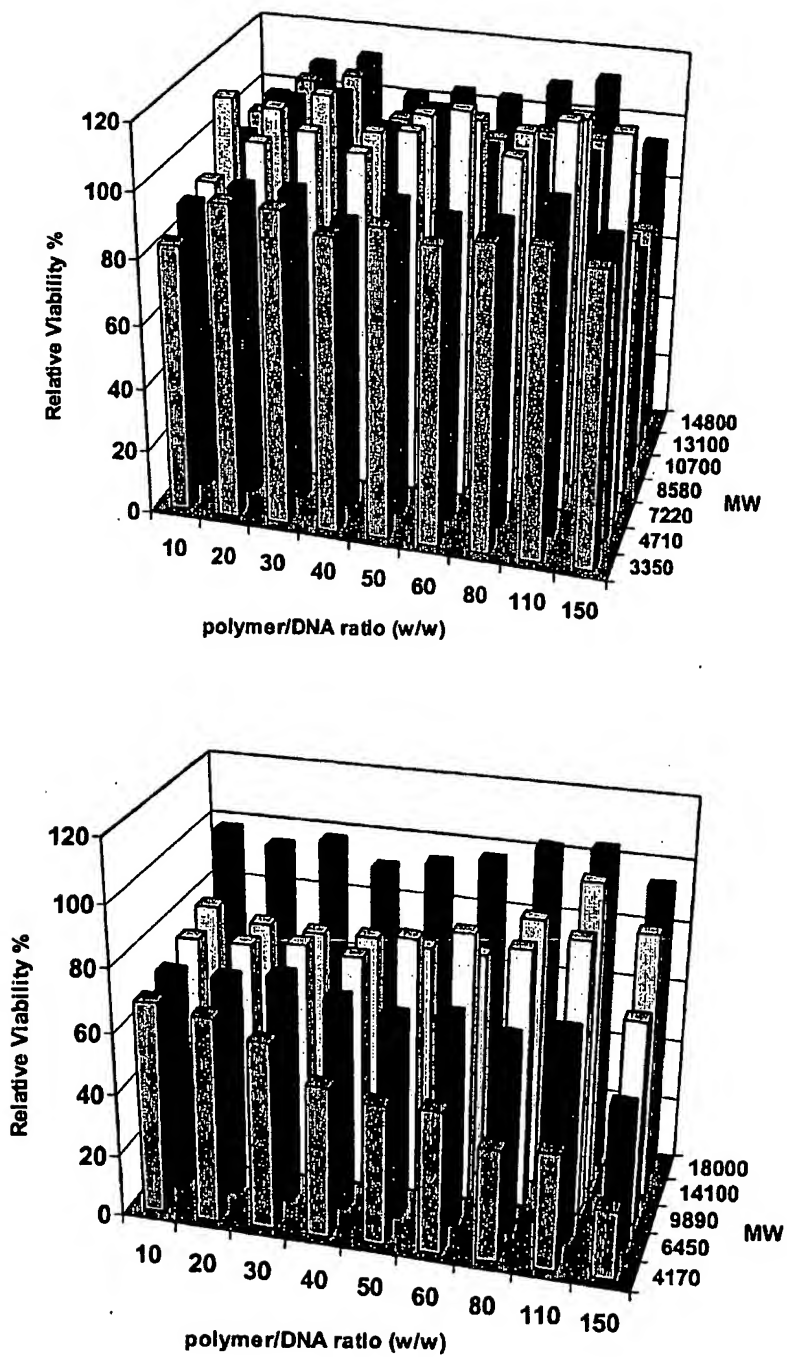
Figure 18

Figure 19

20/30

Figure 20

Figure 21

Figure 22

Figure 23

Figure 24

Figure 25

Figure 26

Figure 27

Fig. 28

Fig. 29

Fig. 30